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Localization and Mutational Analysis of the Nuclear and Aggregation-Prone Ime4 Protein in *Saccharomyces cerevisiae*

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Localization and Mutational Analysis of the Nuclear and Aggregation-Prone Ime4
Protein in *Saccharomyces cerevisiae*

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biological Sciences

by

Patricia Dehon

B.S. Louisiana State University

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During my time at the University of New Orleans, I have learned more than I can ever imagine and am on a whole other level of knowledge. I started the program having no research experience with the exception of undergraduate courses. When I look back at my first semester, I realize I have learned a whole new way of thinking and have become much more confident in myself as a result.

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TABLE OF CONTENTS

LIST OF TABLES	iv
LIST OF FIGURES	v
ABSTRACT	vi
INTRODUCTION	1
MATERIALS AND METHODS.....	11
RESULTS	28
DISCUSSION	59
REFERENCES	73
APPENDIX.....	77
VITA.....	78

LIST OF TABLES

Table 1: Growth media used in this study	22
Table 2: Yeast strains used in this study	23
Table 3: Plasmids used in this study	24
Table 4: Primers used in this study	25-26
Table 5: Results of Mutational Analysis.....	57

LIST OF FIGURES

Figure 1: Ime4 induction in response to pheromone	29
Figure 2: Pheromone sensitivity in BY4741 and a strain containing the mutant $\Delta ime4$...	31
Figure 3: Ime4 localization during pheromone induction.....	33
Figure 4: Ime4-GFP signal with and without ethanol.....	34
Figure 5: Nuclear localization of Ime4 in response to pheromone	35-36
Figure 6: P-bodies in a strain containing Dcp2-RFP	37
Figure 7: Co-localization of Ime4 and P-bodies	38
Figure 8: Percentage of cells with P-bodies in wild-type BY4741 and $\Delta edc3$	40
Figure 9: Percentage of wild-type BY4741 and $\Delta edc3$ cells with Ime4 aggregates	41
Figure 10: Percentage of cells with P-bodies in cells containing YEp352 and pJS14	43
Figure 11: Schematic of <i>pYEpCYC1</i> and <i>YEpCYC1-IME4-GFP</i>	45
Figure 12: Fraction of sporulating cells in cells in the wild-type vs. strain containing the <i>CYC1-IME4</i> construct	46-47
Figure 13: Affect of Ime4 overexpression on the mating response	48
Figure 14: Pheromone sensitivity in a yeast strain containing <i>pYEpCYC1</i> vs. <i>YEpCYC1-IME4-GFP</i>	49
Figure 15: Nuclear localization and inclusion formation of <i>CYC1</i> -driven Ime4.....	51
Figure 16: Stability of Ime4 following cycloheximide treatment.....	53
Figure 17: Western blot showing stability of Ime4 following cycloheximide treatment	54
Figure 18: Schematic and mutational analysis of amino acid regions of <i>YEpCYC1-IME4- GFP</i>	56

ABSTRACT

In *Saccharomyces cerevisiae*, Ime4 is a protein that is induced during meiosis and has a primary role in regulating sporulation in starving diploids. One function of Ime4 is methylation of adenosine residues within mRNA transcripts. Recent studies have shown Ime4 to be induced in haploids during the mating response, although its role in mating has not been determined. In this report, I identify the subcellular localization of Ime4 during the mating response through treatment with alpha factor. A plasmid containing *IME4-GFP* under the control of the medium strength promoter *CYC1* was created in order to express the protein in a controlled manner. Lastly, mutational analysis was conducted to determine which regions of the protein were necessary for its nuclear localization, aggregation, and sporulation function.

Keywords: Ime4, meiosis, sporulation, methylation, mating, *CYC1*, mutational analysis, aggregation, nuclear localization

INTRODUCTION

Saccharomyces cerevisiae Life Cycle

The budding yeast *Saccharomyces cerevisiae* exists in three forms, *MATa* (a cell) and *MAT α* (α cell) haploids that mate to form the third cell type, *MATa/ α* (a/ α cell) diploid. All three types can grow mitotically by budding and have a eukaryotic cell cycle consisting of G1, S, G2, and M phases. Under certain conditions, the diploids undergo meiosis, and the haploid nuclei are packaged into dormant spores. Finally, both haploid and diploid cell types can grow in a filamentous form, forming pseudohyphae. All of the developmental alternatives are controlled by a combination of genetic and environmental signals (Bardwell, 2004).

Saccharomyces cerevisiae mating

The mating process is a highly regulated process involving numerous steps to control growth. The process begins with the recognition of pheromones by one of about 10,000 specific pheromone surface receptors present on the surface of haploid cells. Both a and α cells produce mating factors that are recognized by the opposite cell type. For instance, a cells produce a-factor, a twelve-residue peptide attached to a lipid group, that is recognized by α cells; α cells produce α -factor, a thirteen-residue peptide that is recognized by a cells. Synthetic α -factor is used to induce the mating response of a cells because a-factor more difficult to synthesize and purify and sticks to most surfaces. Once recognition occurs, the cells stop dividing in G1 phase of the cell cycle and begin transcribing genes specific for mating. In response to pheromone, the expression of two hundred genes changes, which composes about 3% of the entire genome (Bardwell, 2004). This includes 100 genes that are repressed and others that are expressed. The cells then begin to grow towards each other; this polarized growth is known as a shmoo formation. Growth is towards the highest amount of pheromone; therefore, it is chemotrophic.

Proteins involved in signaling, polarization, cell adhesion and cell fusion localize to the projection. The cells are then able to make contact and undergo cellular and nuclear fusion to form the a/α diploid cell. The diploid can then undergo meiosis to form haploids or mitosis to duplicate into more diploids, depending on nutritional conditions. Mating responses do not occur in the a/α diploids because the $a1/\alpha2$ heterodimeric repressor present in these cells leads to repression of haploid specific genes, including those encoding the pheromone receptors. Negative feedback methods to prevent constant activation of the pheromone response pathway include degradation of α -factor, ubiquitin-mediated degradation of the pheromone receptor, and degradation of other proteins in the pheromone response pathway (Bardwell, 2004).

Mitogen-Activated Protein Kinase (MAPK) Signaling Pathways

All living cells need a way to sense and respond to changes in the environment. A common way to do so is the use of the mitogen-activated protein kinase (MAPK) module, which consists of multiple protein kinases in a cascade that phosphorylate when activated. An environmental stimulus leads to activation of MAPKKK, which activates MAPKK, which in turn activates MAPK; MAPK then activates transcription factors, translation regulators, and other proteins (Chen, 2007). MAPKs are proline-directed kinases, meaning they phosphorylate serine or threonine that is followed by a proline (Bardwell, 2004). Overall, the MAPK signaling cascade has important roles in regulating metabolism, the cell cycle, gene expression as well as other important cellular processes (Chen, 2007).

In *Saccharomyces cerevisiae*, five MAP kinase cascades are present, including the pheromone-response cascade involved in mating. The pheromone response pathway uses a MAPK cascade to induce mating, using pheromones as the environmental stimuli to begin the cascade leading to activation of a key transcription factor, Ste12. Depending on the cell type,

haploids have either Ste2 or Ste3, which are G-protein coupled receptors that are located within the cell membrane and recognize the specific pheromone of the opposite cell type (Chen, 2007). The G protein is maximally active thirty seconds after addition of pheromone (Bardwell, 2004). Once recognition occurs, the heterotrimeric G protein dissociates, and the active portion activates Cdc42, which is a GTPase. When Cdc42 is active, it is bound to GTP and activates Ste20, which activates the MAPK cascade by autophosphorylation followed by phosphorylation of Ste11 (MAPKKK). Ste5 acts as a scaffold to keep the component of the MAPK pathway in close contact. Ste11 phosphorylates Ste7 (MAPKK), which activates Fus3 or Kss1 (MAPK). Fus3 has the role of activating the expression of genes to prepare for mating. These include genes for cell cycle arrest, polarized cells growth, and nuclear and cellular fusion. Another MAPK that can be activated in the mating response through the same cascade is Kss1. If neither Kss1 nor Fus3 is present, the cell is sterile, but if one of the two is present, the cell can mate. This suggests that the two proteins have a redundant function, although Fus3 is responsible for most of the mating response. Kss1 has an additional role of filamentous growth that Fus3 does not have (Chen, 2007). Both Fus3 and Kss1 act by phosphorylating Ste12, Dig1, and Dig2. Ste12 binds directly to DNA to activate transcription; Dig1 and Dig2 prevent this. Phosphorylation of these components by Fus3 or Kss1 prevents Dig1 and Dig2 from binding and repressing Ste12. Activity of MAPKs is detected within minutes of pheromone addition, and changes in gene expression are noted within 15 minutes (Bardwell, 2004).

A MAPK pathway used in filamentation is inhibited in part through the action of Mpt5, an RNA-binding protein that binds the 3' region of mRNAs of several genes involved in filamentation. When the gene encoding Mpt5 is deleted, the MAPK pathway for filamentation is derepressed. The tight regulation of the pathway is due to post-transcriptional control by the

critical RNA-binding protein Mpt5. As a result of the action of Mpt5, cell wall integrity, recovery from mating pheromone arrest, and stress tolerance is possible (Prinz, 2007).

Initiation of Meiosis and Sporulation

The initiation of meiosis is a highly regulated process involving a cascade of events and interlocking pathways. Only diploids under carbon and nitrogen starvation conditions are able to activate meiosis because the starvation conditions do not allow for mitosis to occur. The $a1/\alpha2$ repressor that is present in diploids leads to repression of *RME1*, which encodes a protein that inhibits *IME1* expression and thereby prevents initiation of meiosis. *IME* genes and proteins are “initiators of meiosis”. Ime1 is a transcription factor that is expressed at high levels in starved *MATa/MAT α* diploids. Ime1 activates *IME2* and other genes for sporulation and meiosis. *IME2* encodes a protein kinase. The whole pathway is controlled in part by Ime4, which is at high levels in starved diploids. Without Ime1, sporulation cannot occur, and the *IME1* transcript relies on Ime4 to accumulate; therefore, both Ime4 and Ime1 are the essential precursors for the induction of meiosis and sporulation (Shah, 1992). More than five hundred genes are turned on as a result of this cascade. Ime4 has N6-methyladenosine activity, a post-transcriptional modification, used to regulate the genes for meiosis. Methylation of various transcripts by Ime4 is necessary for meiosis and sporulation. This includes methylation of *IME1* and *IME2* mRNAs, as well as *IME4* mRNA itself (Bodi, 2010). Because only diploids are able to undergo meiosis and sporulation, another method of control used by haploids is antisense RNA. The *a* and α cells produce antisense RNA for the *IME4* transcript; therefore, transcription of *IME4* does not occur. The antisense transcript is shut off in *a/α* diploids by $a1/\alpha2$ (Shah, 1992).

Regulation by Post-transcriptional Mechanisms

Although much regulation of cell type and cell cycle processes is accomplished by differential transcription, there are many examples in which post-transcriptional events play regulatory roles in gene expression. The mRNA transcripts are modified in many ways directly after transcription, including addition of a 5' cap, 3' tail, splicing, and nucleoside modifications. The 7-methylguanosine cap is added to the 5' end of the transcript. The 3' tail consists of multiple adenosine monophosphates that are added to the 3' end of the transcript. In some cases, the transcript can undergo splicing, in which introns are spliced out and exons are ligated together for the final transcript. Then, single nucleotides within the transcript can be modified by adenosine methylation via Ime4 and its orthologs. This methylation is the second most common post-transcriptional modification (Wlodarski, 2010). Lastly, the mRNA is exported to the cytosol in the form of a protein-bound hnRNP for eventual translation. In principle, any of these steps can be regulated, with positive or negative effects on the production of the protein.

Another method of post-transcriptional modification is achieved through regulated splicing, which can result in a disruption of translation through frame-shifting or nonsense codons. Juneau (2007) identified thirteen meiosis-specific intron-containing genes in which splicing occurs only during sporulation; incomplete or no splicing occurs during vegetative growth. Microarray analyses showed that an extensive amount of regulated splicing occurs when yeast are grown under sporulation-inducing conditions. This method of post-transcriptional modification during nutrient-limiting conditions is thought to act as another method of control to further repress protein synthesis and premature entrance into meiosis. *MER1* and *NAM8* are genes in yeast that encode proteins identified to regulate splicing in meiosis-specific genes (Juneau, 2007).

mRNA Modification by the MT-A70 Family of Methyltransferases

Recent work in several labs has demonstrated the importance of m6A in developmental and regulatory decisions. The term “RNA epigenome” has been proposed to describe the fact that the presence of m6A at particular locations in a transcript can alter downstream processes and thereby regulate protein production (He C, 2010). Little is known about the precise role of m6A in any case, however.

Internal methylation of mRNA produces N6-methyladenosine (m6A). This modification has been suggested to be universal in eukaryotes and to have various effects on expression, but the precise role is unknown (Bujnicki, 2002). Some possible roles include pre-mRNA splicing efficiency, mRNA export from the nucleus (Bujnicki, 2002), mRNA maturation, structural organization, stability (Bodi, 2010), and protein translation. Methylation can also alter interactions between proteins and nucleic acids (Wlodarski, 2011).

Methylation is always present at low levels throughout the mRNA in humans at an average of one to three residues per mRNA, but the distribution is not random. For instance, some mRNA does not have any methylation (Bokar, 1997). Less than 2% of all adenosines of the eukaryotic pool of mRNA have m6A (Hongay, 2011). Methylation is not just found in mRNA; it is also found in snRNA, tRNA, and rRNA, but methylation of these transcripts does not require Ime4 (Bokar, 1997). Methylation requires enzymes from the S-adenosyl-L-methionine (SAM)-dependent methyltransferase (MTase) superfamily. Methyltransferases act by transferring a methyl group from SAM to a nucleophilic acceptor within proteins, nucleic acids, small molecules, and lipids (Wlodarski, 2011).

The first adenosine methyltransferase for pre-mRNA was identified and purified from a HeLa cell nuclear extract; it was found to be associated with a 70-kD subunit of a 200-kD

protein complex; therefore, it was then named MT-A70. MT-A70 is the founding member of the MT-A70 family of proteins found in virtually all eukaryotic organisms. The remaining members of the complex remain to be identified. A consensus sequence for methylation was identified, RRACH, in which R is a purine, A is the methylation site, and H is A, C or U. The most common consensus is GAC, which is conserved in plants, yeast, and mammals (Bodi, 2010). The most common extended sequence is GGACU. MT-A70 was found to have subnuclear localization in HeLa cells, which suggests a role in pre-mRNA splicing (Bokar, 1997). m6A occurrence in mRNAs has also been correlated with stop codons, suggesting a role in translational control but also with long internal exons within the pre-mRNA. Because m6A is in long internal exons, in genes that have multiple isoforms, and co-localizes with splicing proteins, m6A could have a role in RNA splicing (Dominissini, 2012). These are not mutually exclusive possibilities, however, and it is possible that m6A can have different effects in different contexts.

Phylogenetic analyses identify nucleic acid-directed methyltransferases in various eukaryotes as well as bacteria and some viruses. Most have a common structure consisting of α helices and β sheets; the β sheet contains a cleft where AdoMet binds (Bujnicki, 2002). m6A is found in both DNA and RNA of various species, with a range of roles in each. The catalytic core of the MTase domain resembles DNA MTases of some bacteria. A conserved sequence is noted in the m⁶-adenosine methyltransferase domains across various species, including the human MT-A70, *S. cerevisiae* Ime4, and the *Drosophila* and *Arabidopsis* homologs. If the methyltransferase is deficient, it is detrimental and leads to apoptosis, developmental arrest, or defects in gametogenesis in different systems (Dominissini, 2012).

In *Arabidopsis* plants, the majority of m6A is located toward the 3' end of transcripts close to the poly-A tail and deficiency results in up-regulation and down-regulation of a number

of genes although some of the effects are likely to be indirect (Bodi, 2010). By creating mutants in which expression is reduced, not a knockout, in *Drosophila*, the methyltransferase, *Dm IME4*, was found to be required for viability. The RNA and protein are present in the gonads and has been suggested to have a role in Notch signaling for interaction between the soma and germ line cells; without methylation, the egg chamber does not form (Hongay, 2011).

Inducer of Meiosis (Ime4) Protein

Ime4 was identified genetically as an activator of meiosis (Inducer of Meiosis 4), but it is not a transcription factor, a common method of regulation. It has m⁶-adenosine methyltransferase activity, which is thought to activate Ime1 through modification of its mRNA (Clancy, 2002). An internal adenosine within the transcript is methylated, which is different from cap methylation (Serva, 1998).

Ime4 as an m⁶A-methyltransferase was suggested by various observations, the most compelling of which is the high degree of sequence similarity between the Ime4 protein and human MT-A70 and its other eukaryotic orthologs. Ime4 is a 600-amino acid protein, whereas, MT-A70 is a 580-amino acid protein. The highest similarity between these sequences occurs in the C-terminal region, particularly residues 312-522 of Ime4; almost 95% similarity is noted in this region, which is where the catalytic domain is located. Both proteins also have a conserved MT-A70 signature sequence located at residues 235-280 of Ime4 (Clancy, 2002). Surprisingly, the region that binds Mum2 to form the MIS (Mum2-Ime4-Slz1) complex is not conserved between human MT-A70 and yeast Ime4.

Experimental evidence has confirmed a role for Ime4 in m⁶A formation. m⁶A formation is increased at times when *IME4* transcription is also increased, which is in diploids that are sporulating or in stationary phase; m⁶A is not seen as much in vegetative cells. As stated, Ime4

is not a transcription factor; therefore, this modification could be used to activate meiosis and sporulation. A mutation in the conserved methyltransferase domain of *IME4* stops sporulation. When Ime4 is active, the *IME1*, *IME2* and *IME4* transcripts are all methylated during the process, which reinforces the high level of regulation of the cascade. If Ime4 is inactive, mRNA is not methylated even when cells are in sporulation medium, and sporulation does not occur (Clancy, 2002).

Mum2-Ime4-Slz1 (MIS) Complex

It has recently been demonstrated that Ime4 interacts with the carboxy-terminal regions of Mum2 (muddled meiosis 2) and Slz1 (sporulation-specific leucine zipper 1) to form the MIS complex (Agarwala, 2012). When all three components are present during starvation conditions, m6A RNA methylation is increased when compared to strains that have a mutant in any of the components. Ime4 and Mum2 have a primary role, with Slz1 having a minor role in m6A RNA methylation. The MIS complex is highly conserved throughout eukaryotic evolution. Ime4 is homologous to MT-A70 in humans and also proteins present in *Drosophila* and *Arabidopsis*. *Arabidopsis* also has a *MUM2* homolog, AtFIP37 that is known to interaction with the Ime4 homolog. *MUM2* is also homologous to the *Drosophila* Fl(2)d and human *WTAP-1* (Wilm's tumor associated protein) genes. All *MUM2* homologs are highly conserved in the C-terminal region, which is the region that is thought to interact with Ime4 although human MT-A70 has not been shown to interact with *WTAP-1* (Agarwala, 2012). The MTase region is common to all MT-A70 family members including some divergent species, such as *KAR4*, a member of a different branch of the MT-A70 family in yeast. Other eukaryotes have *KAR4*-like genes, such as the *METTL14* gene in humans. The invariant cysteines and cysteine-rich region N-terminal to the MTase catalytic region is common to all original MT-A70 family members, including Ime4. The

extreme N terminus and C terminus are not conserved, which includes the Mum2-binding domain. Mutational analysis in which the region in *IME4* that binds Mum2 is deleted shows that Ime4 is unable to sporulate when incubated in sporulation media for 48 hours. Therefore, this region is necessary for the MIS complex to form and for the function of Ime4 in sporulation (Agarwala, 2012).

Research Aims

The focus of this thesis was to determine whether Ime4 has a role in the mating response in *Saccharomyces cerevisiae*. One approach to this was to determine the sensitivity of the yeast cells to pheromone using synthetic alpha factor. Pheromone was then used to induce the mating response in BY4741 haploids expressing a tagged version of Ime4. Once the protein showed to be induced, the localization of Ime4 could be determined using a plasmid encoding GFP-tagged Ime4. The strain containing the plasmid could be induced using pheromone and viewed using fluorescence microscopy. Creating a plasmid containing the medium strength promoter, *CYCI*, ligated to *IME4-GFP*, allowed for a more stable induction of the protein. Mutational analysis of the protein was done to determine which regions of the protein were important for the function of Ime4 in sporulation, its nuclear localization, and its aggregation in the cytoplasm.

MATERIALS AND METHODS

All general chemicals were obtained from Sigma-Aldrich. T4 DNA ligase and BspHI were purchased from New England Biolabs. Taq polymerase, PCR nucleotide mix, and all other restriction enzymes were from Promega. High fidelity Pfu (Hercules) polymerase, kits for plasmid minipreps, PCR purification and DNA extraction were from Stratagene/Agilent. PCR primers were purchased from Geno-Sys or IDT. All general media supplies were from Fisher. Yeast drop-out mix and synthetic alpha factor peptide were purchased from US Biological. Alpha factor was dissolved in methanol at a final concentration of 5 mg/mL before use. Monoclonal antibodies, anti-myc 9E10 and anti-GFP KB2 were purchased from Santa Cruz. Anti-PGK rabbit polyclonal antibody was a gift from Z. Liu.

Growth and Transformation of *Escherichia coli*

Escherichia coli cells were grown on LB medium at 37°C. Medium was supplemented with the required antibiotic, ampicillin, for a final concentration of 100 µg/mL. In order to transform DNA to be expressed in *E. coli*, XL1-Blue Competent Cells were used from Agilent Technologies, and the protocol was followed, selecting on LB supplemented with the appropriate antibiotic.

Growth and Transformation of *Saccharomyces cerevisiae* (High efficiency method)

Saccharomyces cerevisiae was grown in YEPD, SC Casamino acids, SC Dropout, or sporulation (SPM) media at 30°C. To transform yeast, a high efficiency method used by Linda Hoskins/Hahn Lab was used (<http://labs.fhrc.org/hahm>). A colony of a yeast strain was inoculated in 5 mL of YEPD and kept at 30°C overnight while shaking. To dilute, the 5mL of overnight culture was added to 45 mL of fresh YEPD and grown for 1 hour. The cells were spun

down for 5 minutes at 3500 g. The cells were washed in 10 mL of sterile water then resuspended in 1 mL of sterile water and transferred to a 1.5 mL microfuge tube. The sample was spun down and the supernatant discarded. The sample was resuspended in 1 mL of sterile TE/LiOAc (made from 10X TE and 10X LiOAc). The sample was spun down and resuspended in 250 μ L LiOAc. 50 μ L of yeast cells were mixed with transforming DNA and 5 μ L of boiled and chilled single stranded carrier DNA (10 mg/mL). 300 μ L of sterile PEG (40% PEG 4000, 1X TE, 1X LiOAc) was added followed by incubation at 30° C for 60 minutes with occasional gentle shaking. 40 μ L of DMSO was added and the sample was gently shaken. Cells were heat shocked in a 42°C water bath for 15 minutes. The sample was microfuged, the supernatant was discarded, and the cells were resuspended in 1 mL 1X TE. The cells were microfuged for 10 seconds and resuspended in 1 mL 1X TE. 50-100 μ L of cells were plated onto selective media. SC casamino acids was used to select for plasmids containing the *URA3* gene. SC without leucine was used for selection of *LEU2*. Plates were incubated at 30° C for 2-4 days.

IME4 Knockout Strain

A strain of *Saccharomyces cerevisiae* containing an *IME4* knockout was constructed using the *Saccharomyces* Genome Deletion Project as a reference (Winzeler, 1999). Primers used in the yeast deletion project were used to amplify the open reading frame, including the *KAN* gene. BY4741 from the yeast deletion project *Δime4:kan* strain was used as the source of the knockout allele. The PCR product was used to transform wild-type BY4741, selecting on YEPD supplemented with G418 (200 μ g/mL) to select kanamycin resistant isolates. Yeast DNA minipreps prepared from the transformants were used to confirm that the wild-type allele was replaced with *kan*. The resulting strain contains the upstream and downstream regions of *IME4*,

but the *IME4* open reading frame is replaced with *KAN*. To confirm the *IME4* knockout, confirmation primers from the yeast deletion project were used.

Halo Assay

A halo assay was done in order to determine any differences in pheromone sensitivities. The method of Manney was used as described by Errede. Desired strains were spread plated onto YEPD plates. Cells were counted using a hemocytometer in order for 10^6 cells to be plated of each sample. A 4 mm sterile disc of Whatmann filter paper was placed in the center of the plate and 4 μ L of 5 μ g/ μ L α -factor pheromone was added to the center of the disc. The plates were grown at 30° C for 48 hours and an image of the plate was taken using ChemiDoc under epi-white illumination. Methanol was added instead of pheromone as a negative control. The diameters of the zones of growth inhibition were measured using Quantity One Analysis Software to determine any difference in pheromone sensitivity between the two strains (Esch, 2006). A t-test was used to analyze the results.

Microscopy

Cells were viewed using the Nikon Eclipse E800 microscope at 100X magnification with immersion oil. GFP localization was viewed using the FITC filter. To view cells tagged with RFP, the TX RED filter was used. To visualize cells stained with DAPI, the UV filter was used. The DIC setting was used to view the shape of the cells. Pictures were taken using the Photometrics CoolSNAP camera. The computer program MetaMorph was utilized to take the pictures and adjust exposure time. Image J was used to view and adjust the color of the images.

Ime4 Localization

In order to determine the localization of Ime4 throughout the mating response, the desired strain was grown in SC casamino acids at 30°C and α -factor pheromone (5 μ g/mL) was added to

the culture for a final concentration of 10 mg/mL to induce the mating response. Samples were taken at various time points and viewed under the fluorescence microscope using the FITC filter in order to view GFP and determine the localization.

DAPI Staining

A 200 μ L sample of yeast cells growing in liquid media at 30°C was taken and 100 μ L of ethanol was added. After one minute, the cells were spun down to form a pellet. The alcohol was then removed and the cells were resuspended in 50 μ L of mounting medium (8% glycerol in 1X PBS, pH 7.5) and 1 μ L of DAPI (1mg/mL). The cells were then viewed on the microscope using the UV filter. In some cases, 5 μ L DAPI was added to a 5 mL of culture and incubated overnight with no alcohol wash for better detection of the GFP signal.

P-body Induction

Yeast strain with mCherry-tagged Edc3 or RFP-tagged Dcp2, P-body components, were used to determine the presence of P-bodies as described by Nissan and Parker, 2008. The transformant carrying the tagged plasmid was grown in SC media at 30°C and a sample was taken and gently spun down. The cells were resuspended in SC without glucose, spun down again and resuspended in SC without glucose. After ten minutes, the cells were spun down and all but 20 μ L of the SC without glucose was removed. The cells were vortexed to suspend, and 4 μ L were placed on a slide for viewing.

Cycloheximide Treatment

The desired strain was incubated at 30°C for one hour to regain normal growth. For each milliliter of culture, cycloheximide was added to a final concentration of 100 μ g/mL. Samples were taken at various time points for viewing on the microscope or for total cell extraction for western blot.

Protein Analysis

Total Cell Extraction for Western Blot (TCA Method)

The “quick-kill” method was used for Western blot experiments. Desired strains were grown in 5-10 mL YEPD at 30°C. All cultures were determined to have similar ODs. 1mL samples of cells were taken at the desired time points and added to 1.5 mL microfuge tubes containing 160 μ L β -mercaptoethanol(bME)-NaOH concentrate (740 μ L water, 74 μ L bME, 185 μ L 10 N NaOH). Samples were mixed and put on ice for 10 minutes. 82 μ L of 100% trichloroacetic acid (TCA) was then added, mixed and left on ice for 10 minutes. The cells were then spun down for 5 minutes to form a pellet. The supernatant was immediately aspirated completely, taking care not to aspirate the pellet. 50 μ L of freshly made 1x SDS loading buffer (34 μ L 3x SDS loading buffer, 10 μ L 1M dithiothreitol (DTT), 6 μ L 1M unbuffered Tris, 50 μ L water) was added to resuspend the cells. If the color was not blue upon addition of SDS loading buffer, 1 μ L of 1M unbuffered Tris was added stepwise until the color changed from yellow to blue. The samples were then boiled at 95° C for 3 minutes and assayed or stored at -20°C until needed (Liu).

Total Cell Extraction for Semi-denaturing agarose gel electrophoresis (SDD-AGE)

Cell extracts were prepared for SDD-AGE as in Alberti, 2009. Cells were grown at 30°C and harvested by centrifugation. The pellet was resuspended in buffer (50 mM Hepes, pH 7.5; 150 mM NaCl; 2.5 mM EDTA; 1% (v/v) Triton X-100) in addition to 1 mM PMSF, 1 X Complete Protease Inhibitor (Roche), and Sigma protease inhibitor cocktail (P8215) at 1:50 dilution. Glass beads (1.75 g/mL) were added to the cells and vortexed at 30 second intervals for 10 minutes to lyse the cells. The cells were viewed on a microscope to ensure proper lysis. The

cells were gently spun down to sediment any debris. The soluble portion was removed and placed in a new microfuge tube. The pellet was resuspended in 150 μ L of lysis buffer. A 200 μ L sample of extract was added to an equal volume of 4 X sample buffer without reducing agent (2 X TAE; 20% (v/v) glycerol; 4% (w/v) SDS; bromophenol blue). The samples were incubated at room temperature for 15 minutes before being loaded on a 1.8% agarose gel (1 X TAE and 0.1% SDS). The running buffer used contained 1 X TAE and 0.1% SDS. The gel was run at 50-100 V then blotted onto PVDF membrane.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Total protein extracts were separated using SDS-PAGE. Depending on well size, 8-15 μ L of extract was added to the well of a 10% SDS gel (10% Tris-HCl Ready Gel Precast Gel from BioRad). The extracts were separated at 70-120 Volts in 1X SDS gel running buffer (25 mM Trizma Base, 200 mM glycine, 0.1% SDS). Gels were run until the blue band reached the end of the gel. Gels were then transferred to a membrane for use in western blots.

Western Blot

The SDS gel containing the separated proteins, the PVDF membrane cut to size, and two pieces of extra thick filter paper were allowed to soak in transfer buffer (25 mM Trizma Base, 14.4% glycine, 0.05% SDS, dissolved in 80% deionized water and 20% methanol) for fifteen minutes to equilibrate before the transfer of the gel to the membrane. Assembly of the transfer apparatus was done following the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell Instruction Manual (BioRad). Transfer was done at 15 Volts for 30 minutes. The membrane was allowed to dry, rewet in methanol for 10 seconds followed by distilled water for 5 minutes. Non-specific sites were blocked in blocking buffer (5 g nonfat dry milk, 0.1 mL Tween, 100 mL 1x PBS) for one hour. The membrane was washed in 1X PBS-Tween (11.5g/L Na_2HPO_4 (dibasic),

2.96g/L Na₂HPO₄ (monobasic), 5.84g/L NaCl, 0.1% Tween; pH 7.5) for 5 minutes. The primary antibody was diluted 1:200 in PBS-Tween and blocking buffer and incubated overnight at 4°C while mixing. The membrane was washed in PBS-Tween twice for 10 minutes. The secondary antibody was diluted 1:6000 in PBS-Tween and incubated with the membrane for one hour while mixing. The membrane was washed three times in PBS-Tween for 10 minutes each before being detected. Proteins on the membrane were then detected using ECL (Amersham). An equal volume of each solution was mixed and poured onto the protein side of the membrane and incubated for 1 minute. Excess detection reagent was drained, and the membrane was wrapped in SaranWrap. ChemiDoc was used to detect chemiluminescence, and images were viewed and saved using Quantity One Software. Phosphoglycerate kinase (PGK) was used as a loading control.

Capillary Transfer

Transfer for SDD-AGE was done using a transfer method typical for southern blots. A piece of Whatman 3MM Chromatography paper was cut and soaked in transfer buffer before being placed over the gel platform with the ends soaking in transfer buffer. The agarose gel was placed on top of the paper followed by the PVDF membrane cut to size and pre-soaked in transfer buffer. A large stack of dry extra thick filter paper was placed on top of the membrane. The apparatus was compressed with a beaker of water. Transfer took place for 24 hours before disassembling the apparatus and using the membrane for a Western blot.

DNA Manipulations

Restriction Digest

A 20 µL reaction was used for all restriction digests, including 14.5 µL of water, 3 µL of DNA, 2 µL of appropriate buffer, and 0.5 µL of the restriction enzyme, which is added last. All

are added together and incubated at 37 °C for 30 minutes. The restriction digests were analyzed using agarose gel electrophoresis. Some reactions were stored for later use.

Small-scale Plasmid DNA Preparation “Minipreps”

The QIAprep Miniprep Kit was used to purify plasmid DNA, and the protocol for “QIAprep Spin Miniprep Kit Using a Microcentrifuge” was followed except that elution was done using 70 µL of 1X TE pH 7.5.

Agarose Gel Electrophoresis

DNA samples were analyzed using agarose gel electrophoresis. A 0.80% gel was used; therefore, 0.16 g of agarose was dissolved in 20 mL of 1X TBE (100 mM Trizma base, 88 mM boric acid, 1 mM EDTA) in a flask by gently heating in a microwave followed by swirling. The liquid gel was allowed to cool for a few minutes before pouring into the gel apparatus. Combs were inserted to form the wells before the gel solidified. The gel was covered in 1X TBE buffer before loading the gel. To load the gel, 5 µL of loading buffer mixed with 3 µL of the DNA sample was pipetted into the well. 8 µL of the ladder was added in one well as a size reference. The gel was then run around 130 volts. In order to view the bands, the gel was soaked in ethidium bromide for 10-15 minutes and viewed under UV light. A picture of the gel was taken using Quantity One software.

Sequencing Preparation

A 6 µL reaction mix of 5 µL of the plasmid (approximately 1 µg) to be sequenced and 5 pmol of primer was prepared. Sequencing was performed by the Keck facility. Sequences were visualized using FinchTV from Geospiza. The sequence was examined using BLAST against the reference genome sequence at the *Saccharomyces* Genome Database (www.yeastgenome.org).

Polymerase Chain Reaction (PCR)

50 μ L reactions were performed to amplify DNA. The components included 5 μ L of 10X Taq PCR reaction buffer that has Mg^{++} already added (1.5 mM final concentration), 1 μ L of 10 mM dNTP mix (200 μ M final concentration), 100 pmol of the forward primer, 100 pmol of the reverse primer, a 1 μ L or 5 μ L sample of DNA to be amplified, 0.5 μ L of 10X Taq polymerase added last, and sterile water to bring the reaction mix to 50 μ L. The reaction mixes were then amplified using the correct parameters. Standard reaction parameters for Taq reactions were initial denaturing, 94°C 3 minutes followed by 30 cycles of 94°C, 30 seconds; 55°C, 30 seconds; 72°C, 30 seconds to 3 minutes depending on the length of the extension product; final extension, 72°C, 10 minutes; hold at 15°C. The PCR product was then confirmed using agarose gel electrophoresis and was purified and stored.

Polymerase Chain Reaction (PCR) with Hercules

The components of the 50 μ L reaction mix were added as suggested by the Hercules Enhanced DNA Polymerase Instruction Manual (Agilent Technologies). The parameters for amplification were the same as a PCR using Taq except the extension temperature was 68°C. The length of the extension was 1 min/kb of template.

PCR Purification

PCR products were purified to remove reaction components, such as primers, nucleotides, polymerases, or salts. The QIAquick PCR Purification Kit from Qiagen was used following the protocol given. In the final step, to elute the product, 1X TE pH 7.5 was used as elution buffer.

CYC1 Plasmid Constructions

Yeast plasmid *pLGΔ312* was digested with EcoRI and confirmed by running on a 0.8% agarose gel. The region starting from -321 was then amplified by PCR using the *CYC1* forward and reverse primers. The forward primer was designed to contain an EcoRI site, and the reverse primer was made to contain BspHI, SacI and PstI sites for cloning purposes. The BspHI site was picked because it contains an ATG site; therefore, the whole *IME4* gene can be cloned into that site and be in frame without being altered. The PCR product was then purified using the PCR purification kit protocol (Qiagen). The resulting plasmid contains an EcoRI site 321 nucleotides upstream from the *CYC1* transcript start site, the *CYC1* promoter, 5' UTR and ATG, and BspHI, SacI, and PstI sites were incorporated at the 3' end in the PCR reaction. The *CYC1* PCR product and *YEp352* were restriction digested with both EcoRI and PstI. The products were then purified following the PCR purification kit. The cut products were then ligated using a 40 µL reaction mix, consisting of 1 µL ligase, 10 µL of *CYC1* product, 5 µL of *YEp352* product, 4 µL ligation buffer, and 20 µL water. The ligation mixture was placed in a water bath inside a Styrofoam box and incubated at 4°C overnight. The ligation product was transformed into *E. coli*. The products in the resulting miniprep DNAs were analyzed. The results indicated that the *CYC1* promoter had been introduced into the *YEp352* backbone, but additional BspHI sites were present.

Examination of the *YEp352* sequence revealed BspHI restriction sites that needed to be removed by site-directed mutagenesis. Three sets of primers, 3009 forward and reverse, 3111 forward and reverse, and 4122 forward and reverse, were designed to bind the *YEp352* backbone and used in a PCR reaction with a long extension time. The protocol for Hercules Enhanced DNA Polymerase “Quick Change” mutagenesis (Agilent Technologies) was used. The correct concentration of template (10-50 ng) was determined by using a nanodrop. The PCR products

were digested with Dpn1 in order to remove the template, and the mixture was transformed into *E. coli*. The products and original were digested with BspHI to determine if the sites were removed. Two of the three sites were removed. The PCR reaction was repeated using the necessary primers, 3009 forward and reverse, to remove the BspHI site at 3009 that still remained. The resulting plasmid was named *pYEpCYC1. IME4* was cloned into *pYEpCYC1* as a PCR product to get a GFP-tagged Ime4 strain with the medium-strength *CYC1* promoter. The *IME4* allele from *IME4-GFP* was amplified by PCR using a forward primer that contains BspHI at the *IME4* start and a vector primer (pGP reverse). The PCR product from *IME4-GFP* and the *pYEpCYC1* construct were restriction digested using BspHI and SacI followed by ligation. The product was then transformed into *E. coli*. The transformation was confirmed by restriction digest using BspHI and PstI followed by running on a 0.8% agarose gel. The plasmids recovered from *E. coli* were re-named *YEpCYC1-IME4 (1-600)-GFP* and transformed into yeast strain BY4741 or YYF101.

Construct of IME4 Deletion Plasmids

Yeast plasmid *YEpCYC1-IME4-GFP* was restriction digested with EcoRI to linearize the circular plasmid. The plasmid was incubated at 65°C for 20 minutes to remove any remaining enzymes from the digest. The plasmid was then diluted 1:100 to be used for PCR. Various primers were used to delete a region of the protein (Table 3). The PCR products were PCR purified and run on a 0.8% agarose gel in 1X TBE to confirm the product. The product was then restriction digested with BspHI and SacI. The digested product was then ligated to the *pYEpCYC1* promoter region. The ligation product was transformed into *E. coli*. The transformants were incubated in LB containing ampicillin liquid media for minipreps. The

product was then transformed into yeast strains BY4741 and YYF101 and plated on SC casamino acids.

The *pYEpCYC1* promoter region was isolated from *YEpCYC1-IME4-GFP* using gel extraction. *YEpCYC1-IME4-GFP* was restriction digested with BspHI and SacI. The product was incubated at 65°C for 20 minutes to remove any remaining reaction components. The product was then PCR purified and run on a 0.8% agarose gel in 1X TAE. Multiple lanes were loaded; the gel was cut and half was stained with ethidium bromide and subjected to UV light to view the bands. The *pYEpCYC1* band was removed and aligned with the unstained gel to extract the band without subjecting it to ethidium bromide or UV light. The gel extraction kit and protocol was followed to isolate the band.

Statistics

Averages, standard deviations and t-tests were done using Microsoft Excel. For the t-test, alpha was set at 0.05. A statistically significant difference is assumed when the p-value is less than 0.05.

Table 1: Growth Media Used in This Study

Medium	Medium Content	Conditions
LB	1% Bactotryptone 0.5% NaCl 0.5% Yeast Extract 2.0 mL 25 mg/mL ampicillin* 1.5% agar pH 7.5 for solid medium	Growth medium to select for <i>Escherichia coli</i> cells containing a plasmid
YEPD	1% yeast extract 2% peptone 2% glucose 2% agar for solid medium	Rich growth media for <i>Saccharomyces cerevisiae</i> cells
SC Casamino Acids	0.67% yeast nitrogen base (without amino acids) 2% dextrose 1% casamino acids 1% adenine 2% agar for solid medium	Nutritional selective media for <i>Saccharomyces cerevisiae</i> cells
SC Dropout	10% glucose 10% 10X dropout mix 10% yeast nitrogen base 2% agar for solid medium 1% amino acid supplements as needed	Nutritional selective media for <i>Saccharomyces cerevisiae</i> cells
Sporulation Media (SPM)	1% potassium acetate 2% agar for solid medium 1% amino acid supplements as needed	Minimal media to induce sporulation

Table 2: Yeast Strains Used in This Study

Name	Description	Source
Z1546	<i>MATa ade2 leu2 trp1 ura3 his3 IME4-18myc:TRP1</i>	R. Young (Harbison, 2004)
Z1256	<i>MATa ade2 leu2 trp1 ura3 his3</i>	R. Young (Harbison, 2004)
<i>edc3</i>	Mutant of P-body protein <i>edc3 MATa</i>	Open Biosystems (Winzeler)
BY4741	<i>MATa</i> mating type <i>his3Δ leu2Δ met15Δ ura3Δ</i>	Open Biosystems (Winzeler)
<i>Δime4</i>	<i>IME4</i> is replaced with <i>kan</i>	Open Biosystems (Winzeler)
YYF101	<i>MATa/MATa</i> ; <i>ime4:TRP1/ime4:TRP1</i> ; <i>ho:HIS3/ho:HIS3</i> ; <i>his3/his3; trp1/trp1</i> ; <i>leu2/leu2; ura3/ura3</i>	Yolanda Fortenberry, M.S. thesis, 1997

Table 3: Plasmids Used in This Study

Name	Description	Source
<i>Edc3</i> (pRP1574)	Edc3-mCherry <i>URA3</i>	Nissan and Parker, 2008
<i>Dcp2</i> (pRP1155)	Dcp2-RFP <i>LEU2</i>	Teixeira, 2005
<i>pRS315</i>	Amp; CEN <i>LEU2</i>	Sikorski and Hieter, 1989
<i>pRS316</i>	Amp; CEN <i>URA</i>	Sikorski and Hieter, 1989
<i>YEp352</i>	High copy plasmid with MCS; 2 μ , amp, <i>URA3</i>	Hill, 1986
<i>YEp352-IME4-GFP</i>	<i>IME4</i> -GFP	B. Huderson, M.S., 2003
<i>YEp352-CYC1</i> promoter	381 bp from <i>CYC1</i> promoter to ATG	This study
<i>YEp352-CYC1-IME4</i> (aa 1-600)- <i>GFP</i>	<i>CYC1</i> ATG fused to <i>IME4</i>	This study
<i>YEp352-CYC1-IME4</i> (aa 120-600)- <i>GFP</i> (Δ K120)	<i>CYC1</i> ATG forward fused to <i>IME4</i> K120	This study
<i>YEp352-CYC1-IME4</i> (aa 216-600)- <i>GFP</i> (Δ I216)	<i>CYC1</i> ATG fused to <i>IME4</i> I216	This study
<i>YEp352-CYC1-IME4</i> (aa 1-545)- <i>GFP</i> (Δ QN)	<i>CYC1</i> ATG fused to <i>IME4</i> with QN-rich C-terminal deletion	This study
<i>pLGA312</i>	<i>CYC1</i> -lacZ, <i>URA3</i> , 2 μ ; amp	Lalonde, 1986
<i>PRM2b</i>	<i>pRS316-IME4</i>	R. Madison

Table 4: Primers Used in This Study

Name	Sequence (5' → 3')	Source/Purpose
IME4 primer D	GTTTCCAGTAAGAACGATTGGAGTA	Confirm Ime4 knockout
kanB	CTGCAGCGAGGAGCCGTAAT	Confirm Ime4 knockout
IME4 primer A	CTTTTGGCGTACAGTATTGTTCTTT	Confirm Ime4 knockout
IME4 full forward	TTTTGAATTCATGATTAACGATAAACTA	Amplify <i>IME4</i>
IME4 full reverse	TTTTGTTCGACCTGAGCAAAATATAGGTT	Amplify <i>IME4</i>
CYC1 promoter forward	GGTTGAATTCAGCAAGATCAAGATGT TTTCA	PCR for plasmid construction
CYC1 ATG reverse	TAAACTGCAGATGAGCTCAATTCAT GATTAATTTAGTGTGTGTAT	PCR for plasmid construction
IME4 Bsp forward	GGGGTCATGATTAACGATAAACTAGTA	<i>IME4</i> containing BspI restriction site
3111 forward	TCAAATATGATCCGCACATGAGACAAT AACCT	Mutagenesis
3111 reverse	AGGGTTATTGTCTCATGTGCGGATACAT ATTTGA	Mutagenesis
3009 forward	TTATAGGTTAATGTCGTGATAATAATGG TTT	Mutagenesis
3009 reverse	AAACCATTATTATCACGACATTAACCTA TAA	Mutagenesis
4122 forward	CTTTTGGATAATCTCGTCACCAAATCC CTTAAC	Mutagenesis
4122 reverse	GTTAAGGGATTTTGGTCACGAGATTATCA AAAAG	Mutagenesis
YEp532 5:7 flank	ACCATGATTACGAATTCGAGCTC	PCR for plasmid construction
Bsp IME4 ATG forward	TTTTATCATGATTAACGATAAACTAGTA	PCR for plasmid construction
K120	TTTTATCATGAAAGTGTCGAGTTTAACA	PCR for plasmid construction

Table 4: Primers Used in This Study (continued)

I216 unaltered	ATCGTTTCTTTACAATGGTCC	PCR for plasmid construction
K120K216 merge	GGACCATTGTAAAGAAACGATTTCTTCAC ATGAAGAACTGGA	PCR for plasmid construction
M523 reverse overlap	ATGGTGATGGTGATGGTGCTGCATAAACT CCTGGTACTT	PCR for plasmid construction

RESULTS

IME4 was first defined as a sporulation-induced gene that is essential for the process of sporulation (Bodi, 2010). Surprisingly, genome-wide studies using microarray showed that the *IME4* transcript is also induced by pheromone in *MATa* cells; therefore, it is not exclusively a diploid-specific gene as was once thought. The induction depends on Ste12 and the MAP kinase cascade. Chromatin Immunoprecipitation (ChIP) analysis, a method used to detect interaction between proteins and DNA, showed detection of Ste12 in the vicinity of the *IME4* promoter (Esch, 2006). Moreover, induction by pheromone depends on upstream components of this pathway as well as the Kar4 transcription factor (Lahav, 2006). Thus, existing information suggested that the pheromone pathway might be a system to study Ime4 localization and function.

Induction of *IME4* by Pheromone

To verify that pheromone induces the Ime4 protein along with *IME4* RNA, a Western blot was done following pheromone induction. The strains Z1546, which expresses Ime4 tagged with a C-terminal 13myc epitope, and Z1256, a control without a myc tag, were grown at 30°C and pheromone was added. Samples were taken before addition of pheromone and every thirty minutes with the last sample being taken at 120 minutes. Samples were immediately added to bME-NaOH to begin total cell protein extraction. The TCA method for total cell extracts was used. Total cell extracts were subjected to SDS-PAGE followed by transfer to a PVDF membrane. The membrane was blotted and probed using a c-myc mouse monoclonal primary antibody (9E10, Santa Cruz) and a goat anti-mouse secondary antibody conjugated with HRP (Jackson Immunologicals). After addition of ECL to the membrane, chemiluminescence was measured using Quantity One Software. The results shown in Figure 1 show that the Ime4

protein is produced in *MATa* cells in response to alpha pheromone as predicted by genome-wide studies of mRNA abundance. The negative control showed no bands. Phosphoglycerate kinase (PGK) was used as a loading control.

Induction by pheromone suggested that Ime4 might be involved in mating. As one approach to address this, whether the absence of *IME4* caused the cells to be more sensitive to pheromone was addressed.

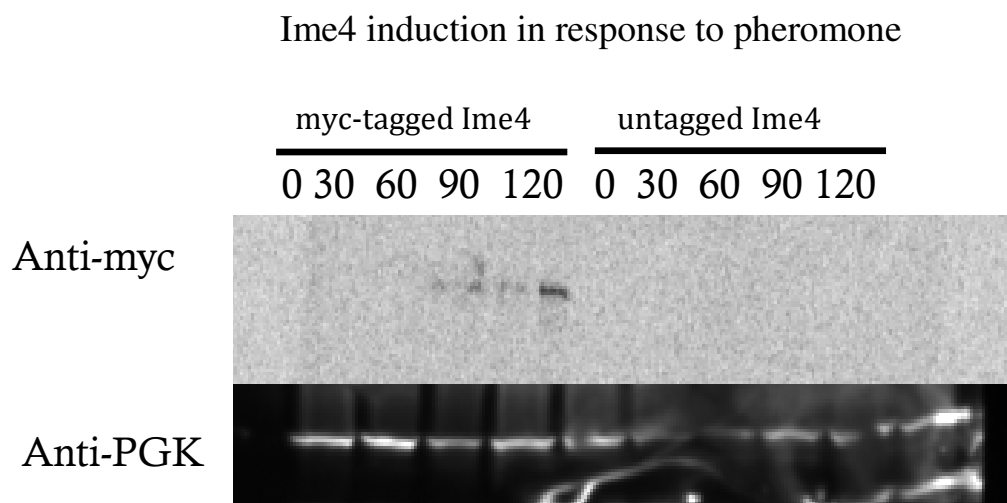


Figure 1: Western blot of myc-tagged Ime4 (Z1546) and untagged Ime4 (Z1256) at various time points following induction with α factor. PGK was used as a loading control.

IME4 Knockout and Pheromone Sensitivity

A strain of *Saccharomyces cerevisiae* was constructed that contains a deletion of *IME4*. A PCR-based method was used as used in the *Saccharomyces* Gene Deletion Project. BY4741 was used as the starting strain. Primers were used to amplify the regions upstream and downstream of the *IME4* open reading frame using the Open Biosystems *ime4:kanMX* strain as a template. The primers contain regions that are homologous to the 5' and 3' ends of the *IME4* gene and were thus expected to amplify the full-length knockout allele from the commercial

strain. The PCR product was then used to transform BY4741, selecting for the G418 (Kan)-resistant colonies. Genomic DNA preparations were then used to confirm the presence of the knockout allele and absence of the wild-type.

In order to determine any difference in the sensitivity to pheromone of a yeast strain containing an *ime4* mutant compared to a wild-type, a halo assay was done (Esch, 2006). The same concentrations of wild-type BY4741 and two mutant strains, *Δime4* 7a and *Δime4* 9a, were plated on YEPD plates. Pheromone was added to a disc of filter paper in the center of the plate. The addition of pheromone halts the yeast life cycle, creating a halo around the disc where cell growth was inhibited (Figure 2A). Methanol was used as a negative control because the α factor used was dissolved in methanol. The plates were incubated for 48 hours at 30° C. An image of the plates was taken and the diameter of each halo was calculated using Quantity One software (Figure 2B). The difference in the sensitivities of the wild-type and mutant strains were statistically insignificant, yielding a p-value of 0.08. The average area of the halo in the mutant was 261.73 mm² and the average area of the halo of the wild-type was 194.31 mm². No halo was evident for the methanol control (Figure 2A). We conclude that the *IME4* gene is not likely required for cell cycle arrest in response to pheromone, as would be indicated by smaller halos in the mutant strains. Conversely, it is not likely required for recovery from pheromone treatment, as would be indicated by larger halos. These roles are not ruled out entirely because Ime4 could have a role that is redundant with another protein involved in the mating response.

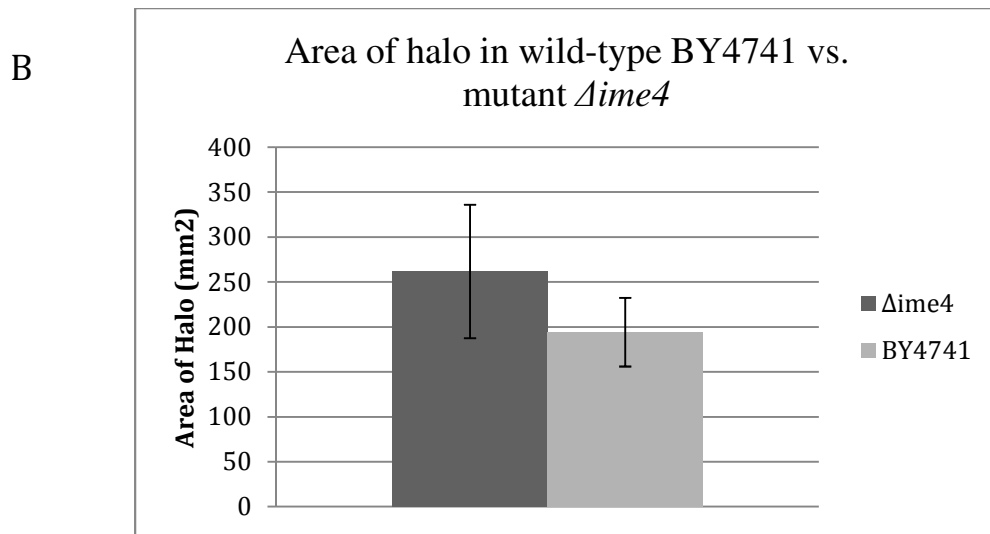
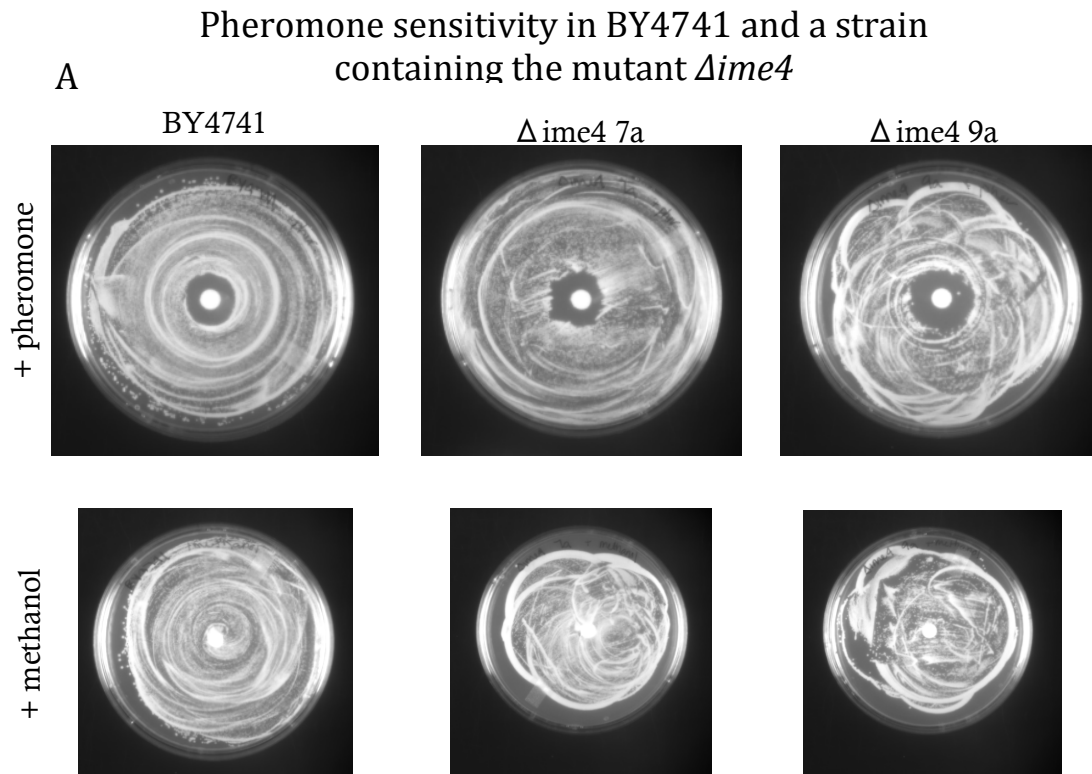


Figure 2 A-B: A Halo assay of BY4741 (wild-type) and $\Delta ime4$ after being plated on YEPD plates with α factor in the center for 48 hours. Methanol was used as a negative control. B. Quantitative analysis of the sensitivities of the two strains to pheromone. The average area of the halo is shown with the standard deviation for error bars. The p-value is 0.08.

Ime4 Localization During Pheromone Induction

Induction with pheromone allowed cellular localization of Ime4 to be easily determined. This differs from induction of sporulation of a/α diploids, which presents difficult technical challenges. The localization of Ime4 following pheromone induction was determined by allowing growth of a yeast strain expressing Ime4-GFP from a high copy plasmid under the control of its own promoter at 30°C in SC liquid media. α -factor was then added and the cultures returned to 30°C to induce the mating response and Ime4 expression. Samples were taken every hour to examine the localization of Ime4 within the cell. Cells were viewed under the fluorescence microscope using the FITC filter. Results shown in Figure 3 show that the distribution of Ime4 changes with time, moving between the nucleus and the cytoplasm and aggregating over time. Shortly after aggregation, the protein disappears entirely. Thus, Ime4 has a dynamic localization pattern, residing in the nucleus and cytoplasm, in aggregates and diffuse in the cytosol.

Ime4 localization during pheromone induction

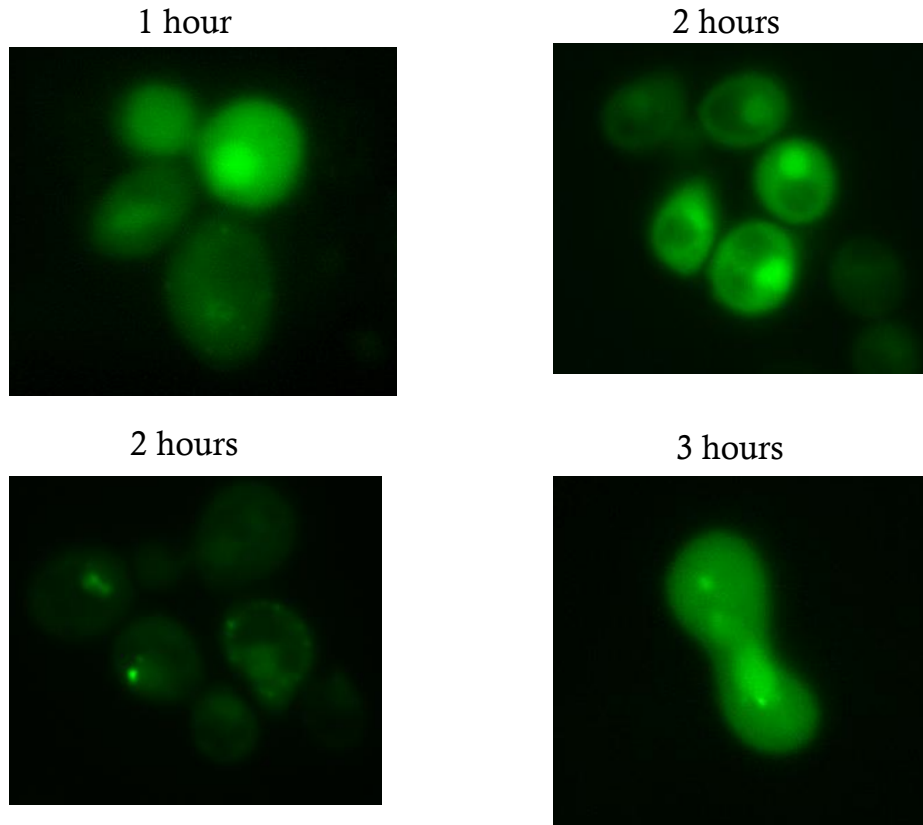


Figure 3: Localization of GFP-tagged Ime4 at various time points following induction with pheromone. The signal is nuclear, cytosolic, and punctate.

Ethanol Wash Control

To determine if the ethanol wash affected the localization of Ime4, the strain was allowed to grow in SC at 30°C for 1 hour. Two samples were taken, one of which was subjected to the ethanol wash while the other was not. In one sample, ethanol was added, spun down, and resuspended in mounting media. The other sample was just spun down and resuspended in mounting media. The samples were viewed on the fluorescence microscope using the FITC filter, and Ime4 localization was compared between the two. The localizations were classified as

nuclear, punctate, diffuse, or a combination of the three. The percentages were calculated out of a total of 154 cells in samples without ethanol and 171 cells in samples with ethanol. Results shown in Figure 4 show no significant difference in the localization of Ime4; therefore, the ethanol wash has no significant effect on Ime4 localization.

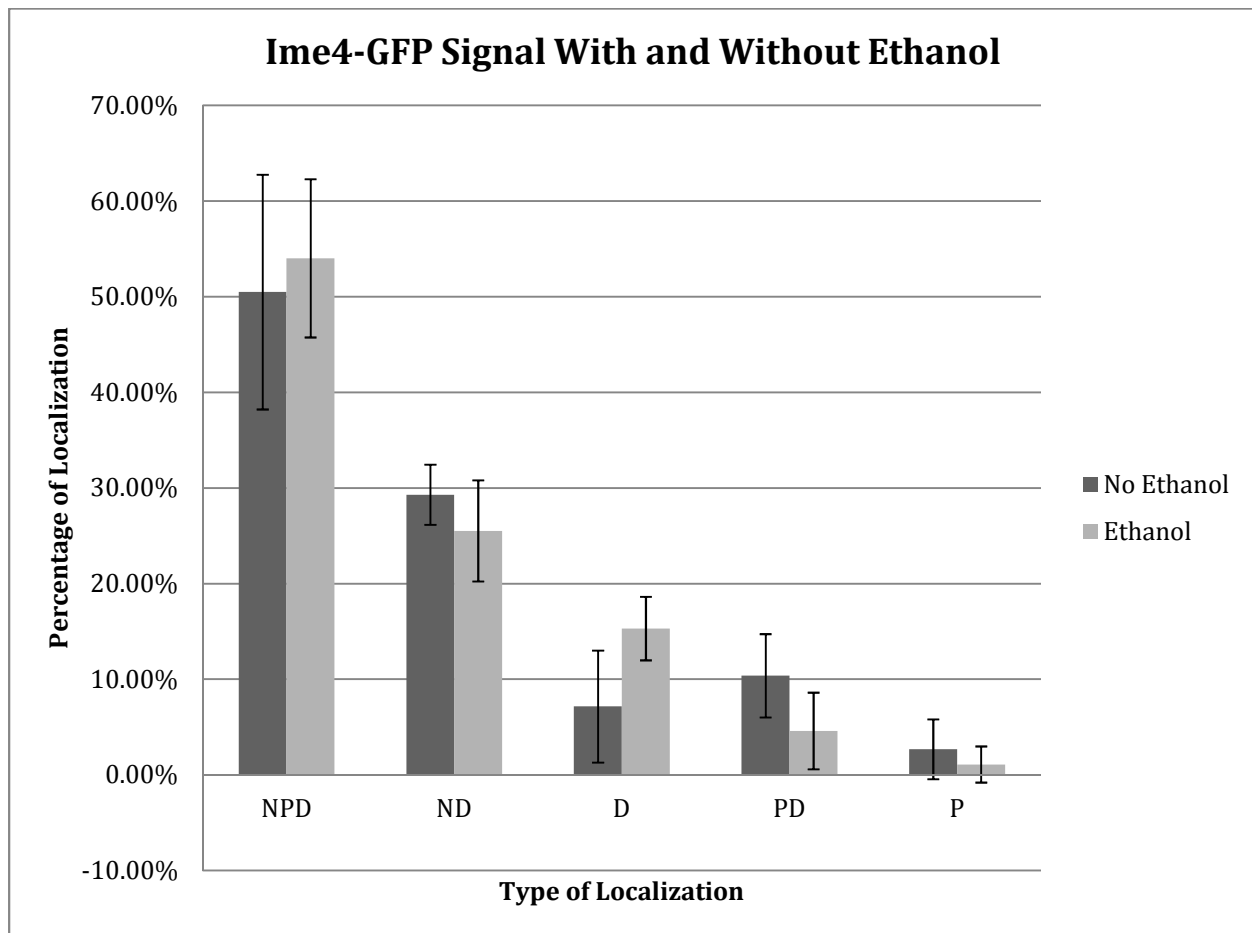


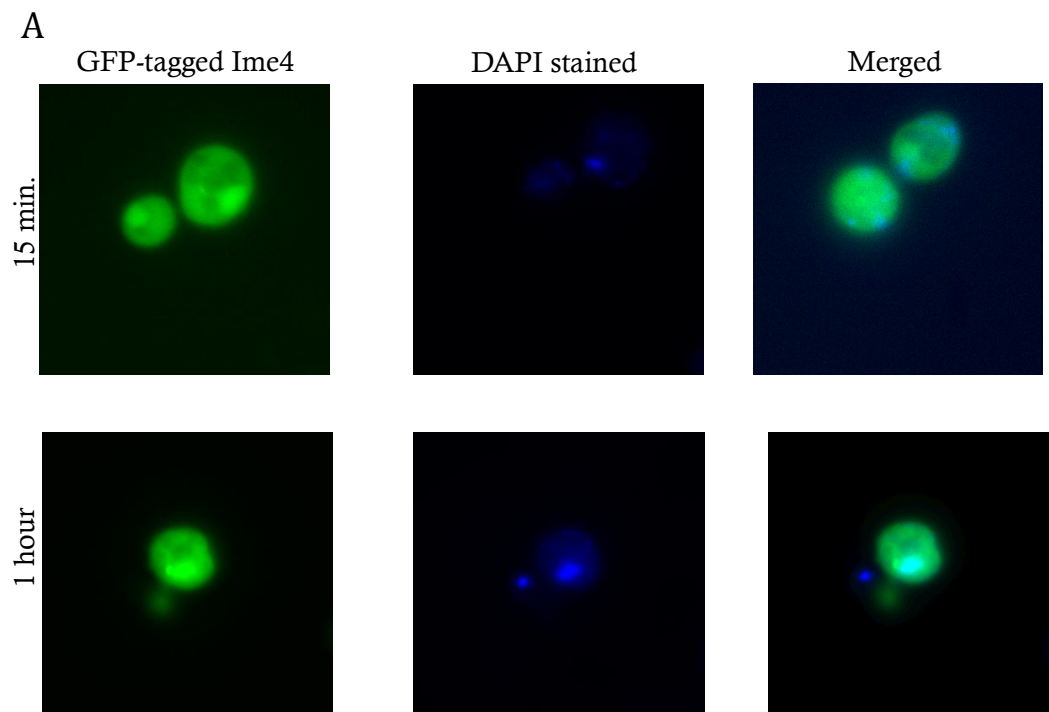
Figure 4: Quantitative analysis to determine any effect of an ethanol wash on the localization of GFP-Ime4. Standard deviations show no difference in the localization between samples treated and not treated with ethanol. N=nuclear; P=punctate; D=diffuse

Nuclear Localization of Ime4 in the Mating Response

In order to determine whether Ime4 actually localizes in the nucleus, as it seems, the experiment was repeated, and the cells were stained with DAPI, a blue fluorescent stain that

binds DNA. The strain containing Ime4-GFP was grown at 30°C in SC liquid media. Following addition of α -factor, samples were taken at various time points. Ethanol was added to a sample of yeast cells, spun down and resuspended in mounting media before the addition of DAPI in order to obtain better nuclear staining. The cells were viewed using the fluorescence microscope. DAPI staining was viewed using the UV filter, and GFP was viewed using the FITC filter. When the two images were merged, colocalization of Ime4 and the nucleus is noted early in the pheromone response as shown in Figure 5. By three hours, the majority of the Ime4 signal had moved to the cytoplasm into aggregates.

Nuclear localization of Ime4 in response to pheromone



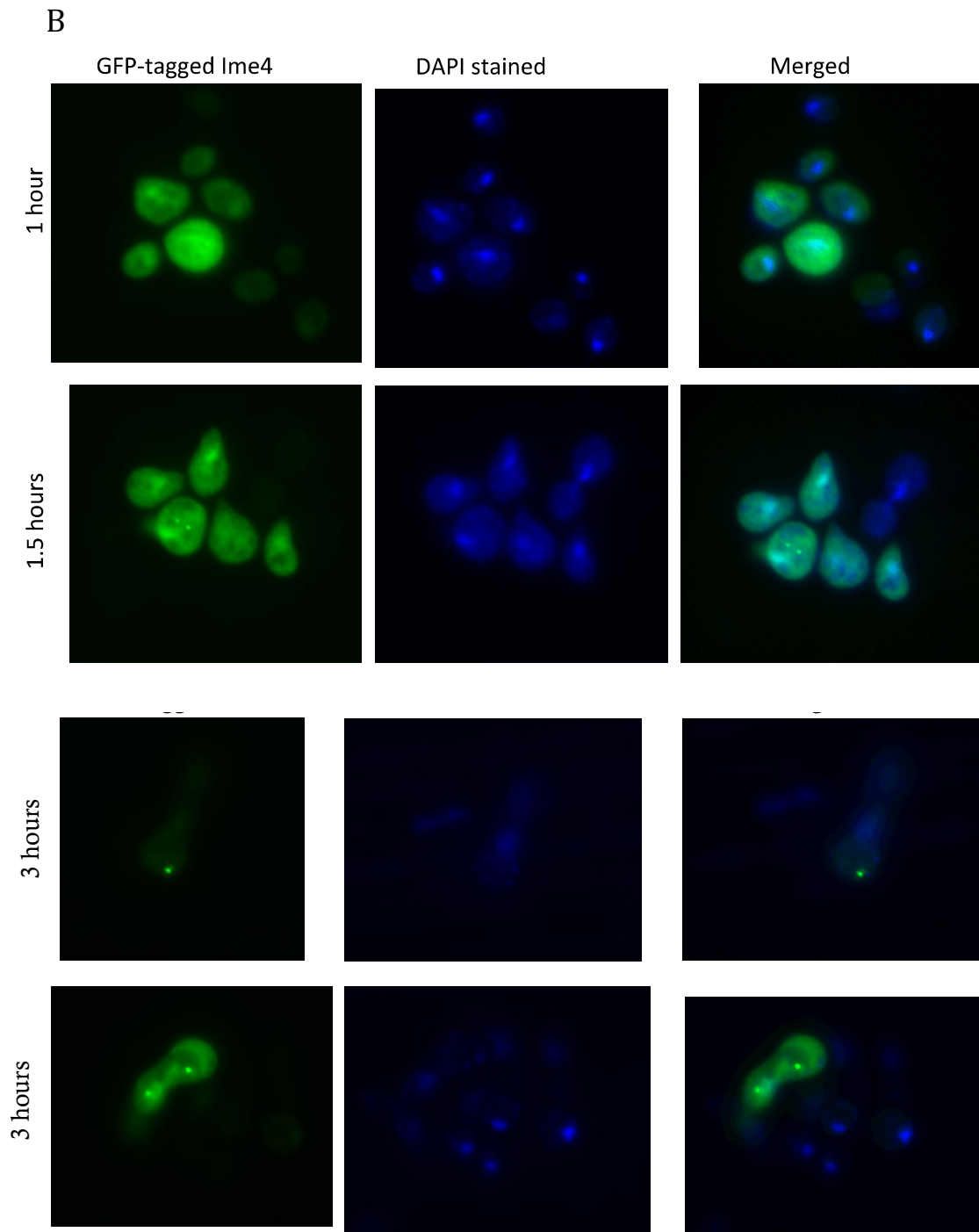


Figure 5: A-B Nuclear localization at various time points following induction with pheromone in GFP-tagged Ime4 after staining with DAPI. The cells have a nuclear signal earlier in the response to pheromone.

P-Bodies and Ime4

A possible reason for the Ime4 aggregates is their localization into P-bodies. To test this, a yeast strain containing an RFP-tagged P-body component, a decapping protein Dcp2, was used (Teixeira, 2005). Induction of P-bodies only was first done in order to ensure proper induction. The yeast strain was grown in SC liquid media at 30°C. A sample was taken, spun down, and resuspended in SC without glucose for 10 minutes. The absence of glucose creates a stressful environment in which P-bodies will form. The cells were then viewed on the fluorescence microscope using the TXRED filter where P-bodies were clearly visible (Figure 6).

P-body induction of a strain containing Dcp2-RFP

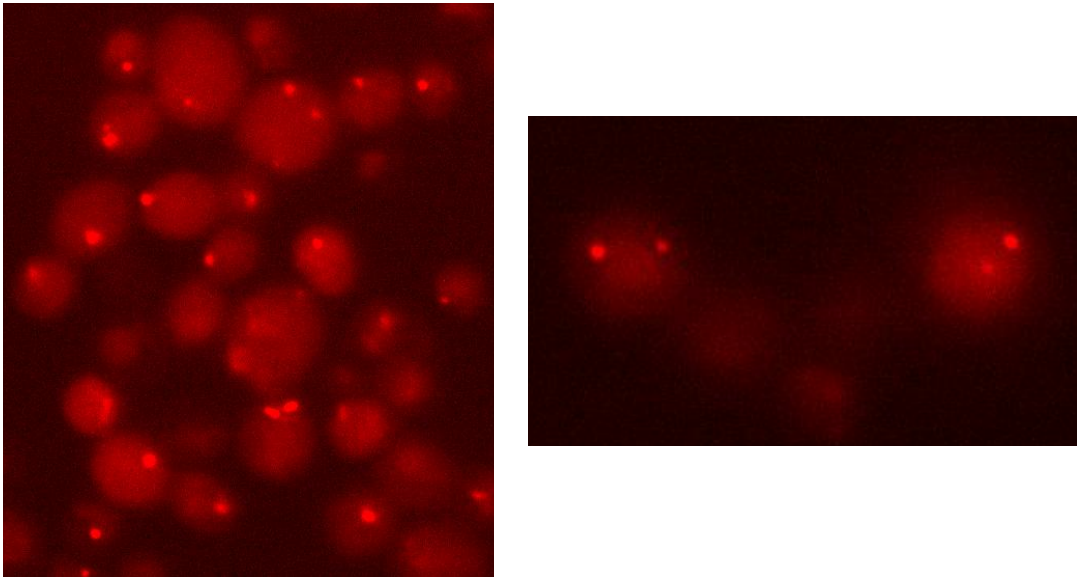


Figure 6: Dcp2-RFP cells after incubating in SC lacking glucose for 10 minutes to induce P-body formation

Once the method of P-body induction was shown to induce P-body production, a strain containing both Dcp2-RFP and Ime4-GFP was used in pheromone induction. The strain was

grown in SC liquid media at 30°C, pheromone was added, and samples were taken at various time points following addition. The cells were viewed on the fluorescence microscope and merged to determine colocalization. Results showed some colocalization, although P-bodies were not present in most cases in which cells contained an Ime4 aggregate (Figure 7). P-bodies were mostly seen in cells containing nuclear Ime4 signals. Therefore, it is possible that P-bodies could prevent the formation of Ime4 aggregates.

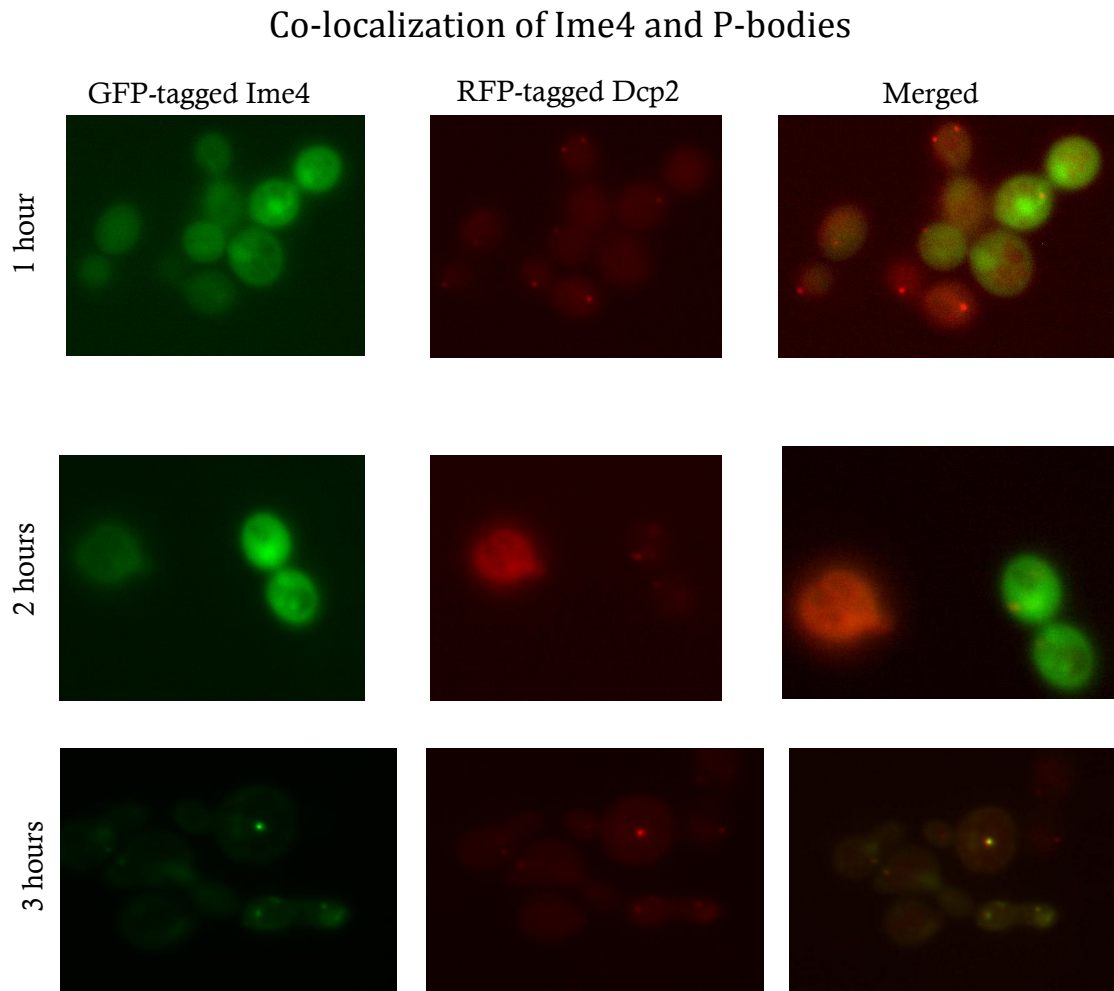


Figure 7: Colocalization of GFP-tagged Ime4 and RFP-tagged Dcp2 at various time points following induction with pheromone.

To test the dependence of Ime4 aggregates on P-body formation, a yeast strain was obtained containing a mutant in the P-body component, *edc3*, an activator of decapping (Wenzeler). With this mutant, the strain should form P-bodies less successfully. To test the ability of the strain to form P-bodies compared to the wild-type, a P-body induction was performed on BY4741 containing Dcp2-RFP and $\Delta edc3$ containing Dcp2-RFP. The two strains were grown overnight in SC –leucine. A sample was taken, spun down, and resuspended in SC –leucine –glucose to induce P-body production. The percentage of cells containing P-bodies in each sample was calculated (Figure 8). The average percentage of P-bodies in the wild-type BY4741 was 58.12% out of 380 total cells compared to 15.98% out of 618 total cells in the mutant $\Delta edc3$. The p-value was 0.0002; because this is <0.05 , a significant difference in the ability to form P-bodies exists between the two strains.

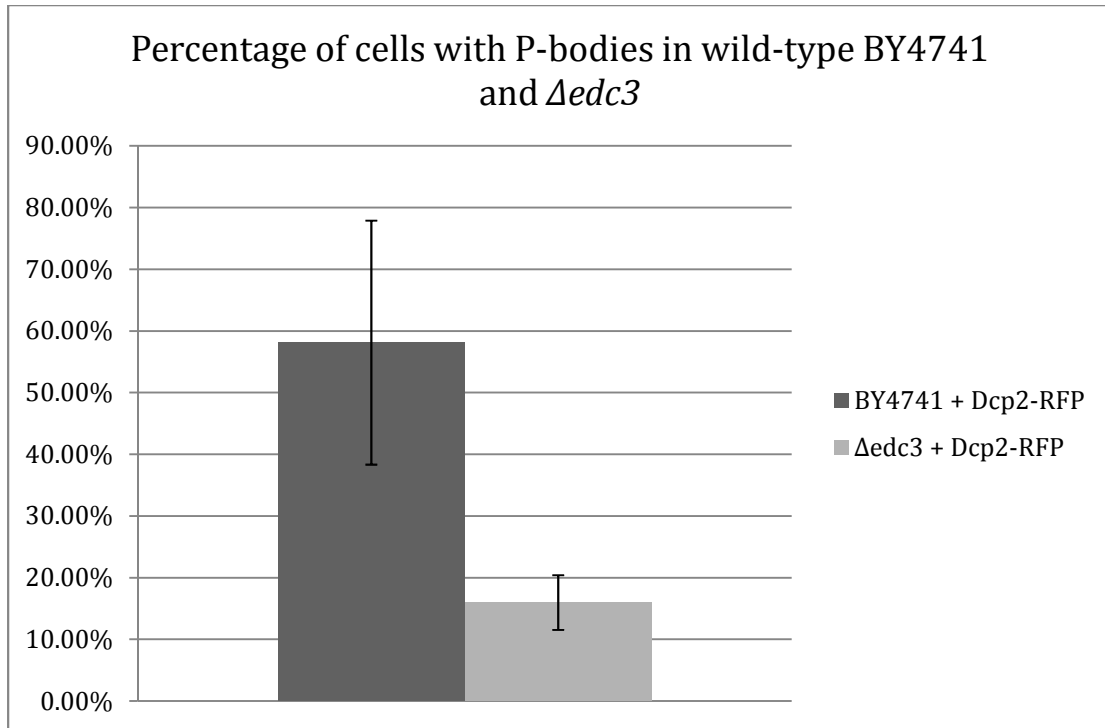
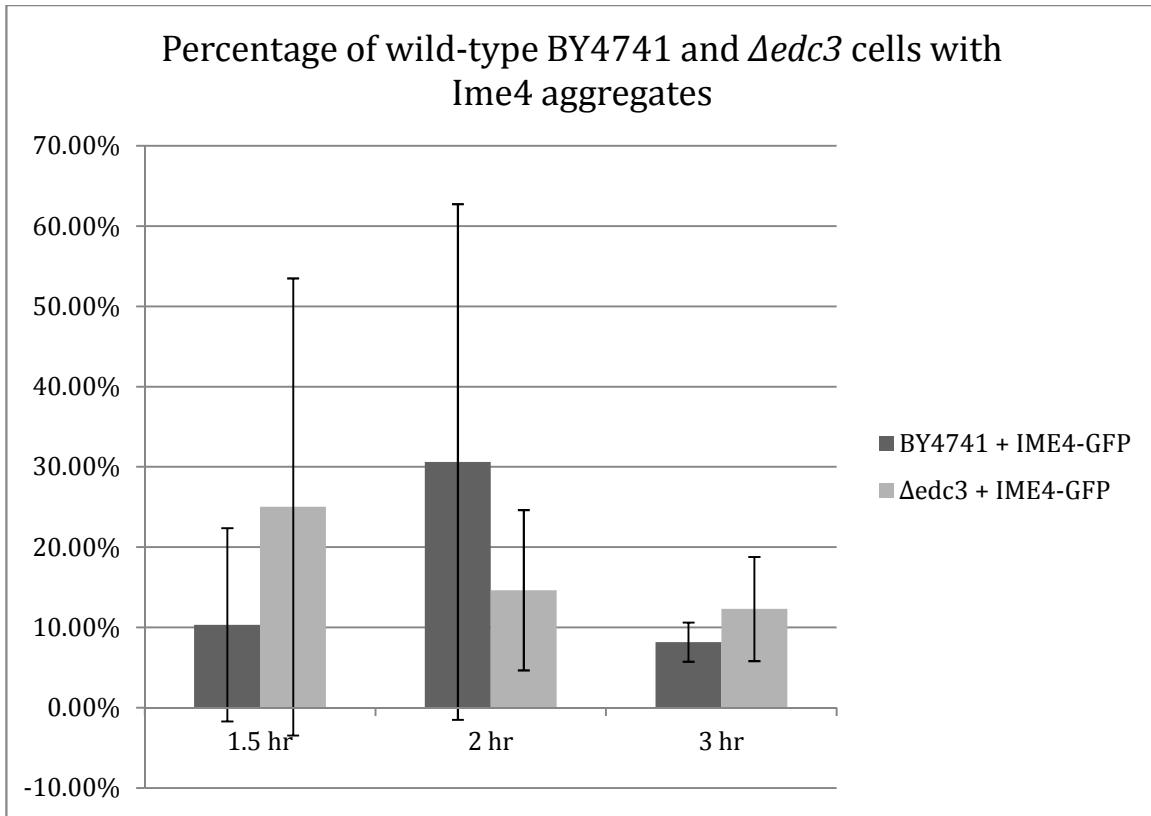


Figure 8: Quantitative analysis of the percentage of P-bodies formed in the wild-type (BY4741) and mutant ($\Delta edc3$) strains with the standard deviation plotted as error bars. The p-value was 0.0002; therefore, a significant difference in the ability to form P-bodies is evident

These two strains, BY4741 and $\Delta edc3$, were then transformed with *IME4-GFP* to determine if Ime4 aggregates depend on P-body formation in order to form. The strains were grown overnight in SC –uracil. Pheromone was added and samples were taken at various time points. The samples were spun down, resuspended in SC –uracil –glucose, and incubated at room temperature for 10 minutes to induce P-body production. Cells were then viewed on the fluorescence microscope using the FITC filter to determine the presence of Ime4 aggregates. The average percentage of cells with Ime4 aggregates was similar in both strains at all three time points (Figure 9); therefore, induction of Ime4 aggregates does not seem to depend on P-body formation.



Time	Ime4 Aggregates in BY4741 + <i>IME4-GFP</i>	Ime4 Aggregates in <i>Δedc3</i> + <i>IME4-GFP</i>	P-value
1.5 hours	32/683	42/235	0.06
2 hours	31/315	21/208	0.17
3 hours	20/276	42/354	0.09

Figure 9: Quantitative analysis of Ime4-GFP aggregates in BY4741 (wild-type) and $\Delta edc3$ at various time points following pheromone addition. Results show no significant difference in the ability to form Ime4 aggregates as shown by the p-values and standard deviation

Another strain was constructed to determine if Ime4 inhibits P-body formation. *pJS14*, a high copy *IME4* plasmid, was transformed into the strain containing Dcp2-RFP. The strain, along with a control of *YEp352*+Dcp2 was grown in SC –leucine –uracil for 1 hour. A sample was taken, spun down, and resuspended in SC –glucose for 10 minutes to induce P-body production. The samples were then viewed on the fluorescence microscope for the presence of P-bodies. An average of 60.67% of 485 cells contained P-bodies in Dcp2 + *YEp352*. An average of 60.78% of 374 cells in Dcp2 + *pJS14* contained P-bodies (Figure 10). The p-value was found to be 0.99; no significant difference was noted in the percentage of P-body formation between the two strains. Therefore, Ime4 does not inhibit P-body formation.

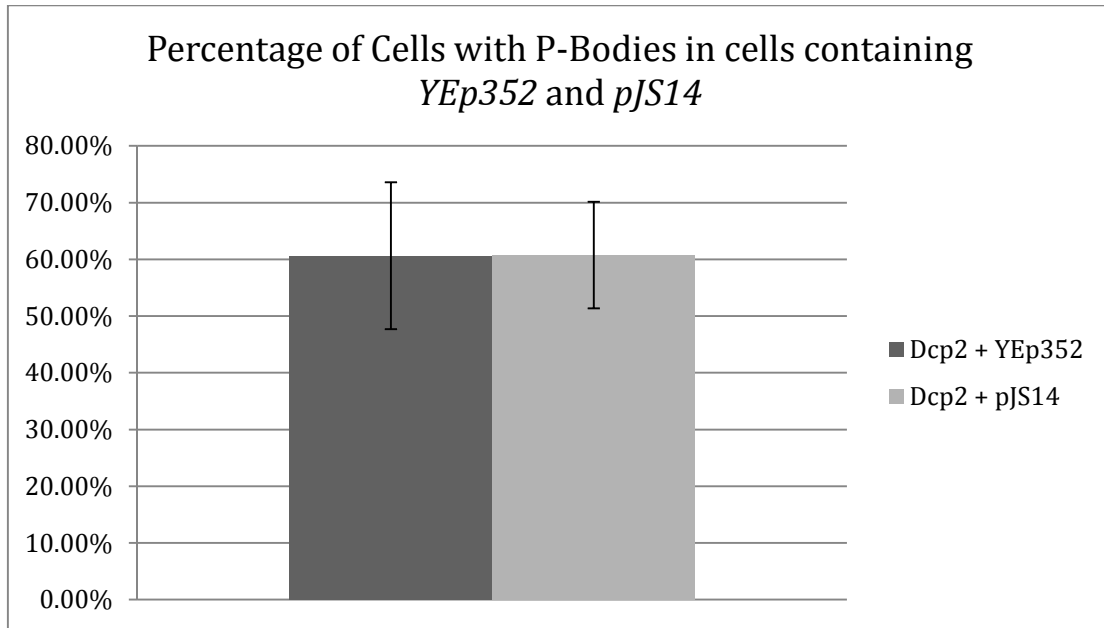


Figure 10: Quantitative analysis of P-body formation in Dcp2 + *YEp352* and Dcp2 + *pJS14* following incubation in SC lacking glucose for 10 minutes to induce P-body formation. Standard deviation showed no significant difference in the ability to form P-bodies.

IME4 Expression Constructs

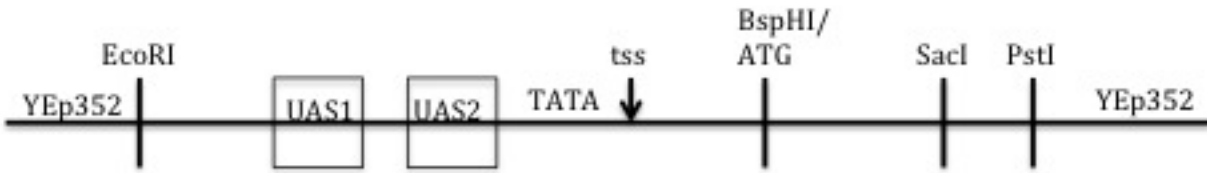
Using the mating pathway to view Ime4 induction is useful, although the protein quickly degrades shortly after the addition of pheromone. Previous work using GAL driven HA and myc-tagged full-length *IME4* showed that the protein is insoluble under these high expression conditions. As a result, it has been difficult to obtain purified protein under non-denaturing conditions; consequently, biochemical approaches have been limited. Therefore, we decided to devise an expression system that is expected to overcome some of the difficulties created by GAL expression systems. One possible mechanism is the use of the *CYC1* promoter. *CYC1* encodes iso-1-cytochrome C, a protein in the electron transport chain that is active during respiration. It contains two upstream activation sequences (UAS) that further control transcription of the gene. It requires heme and the absence of a fermentable carbon source to be

fully active (Lalonde, 1986). We chose to use the *CYC1* promoter because it is medium strength and active during the pheromone response, sporulation, and under conditions in which the Ime4 protein is normally present, including stationary phase and under conditions of nitrogen limitation and acetate carbon sources (spell.yeastgenome.org).

The resulting plasmid includes the *CYC1* promoter, 5' UTR and ATG, and produces full-length GFP-tagged Ime4 within *YEp352*. The desired molecule was constructed in two steps. The first plasmid, called *pYEpCYC1*, contains the two UAS regions and promoter region from the *CYC1* gene, including the TATA box and the ATG codon followed by SacI and PstI cloned between the EcoRI and PstI sites of *YEp352* (Figure 11A). The sequences encoding the restriction sites were included in the primer used to amplify the *CYC1* region from *pLG4312* plasmid. Importantly, the ATG region was modified to include a site for BspHI, allowing inserts to be placed in frame with the *CYC1* ATG without the addition of any extra amino acids. BspHI was chosen above other possible “ATG” enzymes because the recognition sequence allows the *IME4* coding region to be added without any amino acid changes. Site-directed mutagenesis was used to remove the three BspHI sites within the *YEp352* vector.

The second step was to clone *IME4-GFP* between the BspHI and SacI sites of *pYEpCYC1*. The forward primer included a BspHI sequence to permit cloning the coding region of *IME4-GFP* in frame with the *CYC1* ATG. The resulting construct is referred to as *YEpCYC1-IME4-GFP* (Figure 11B).

A



B

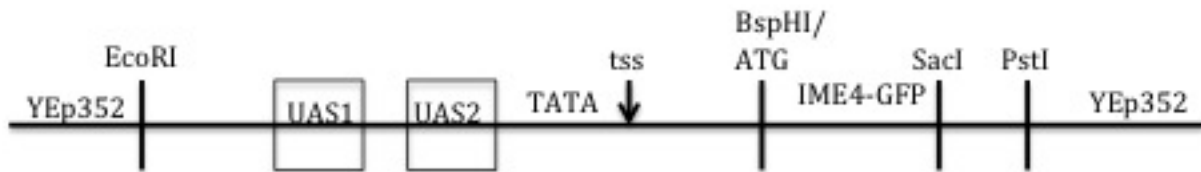


Figure 11: Schematic of A. *pYEpCYC1* and B. *YEpCYC1-IME4-GFP* constructs (not to scale). The *IME4-GFP* addition is about 2.5 kb.

Confirming Sporulation Phenotype

To confirm that the *YEpCYC1-IME4-GFP* construction was functional, it was transformed into the diploid yeast knockout strain, YYF101. In this strain, both resident *IME4* alleles have been replaced by the *TRP1* gene. As a result, the strain is sporulation deficient. *pJS14* is a positive control containing the wild-type *IME4* gene including its own promoter, and *pYEpCYC1* is a negative control because it lacks the *IME4* gene. Once transformed, the strains were grown in SC casamino acids and transferred to SPM media. Samples were taken at 24 hours and 48 hours and viewed on the fluorescence microscope using the DIC filter. Sporulated cells were counted and the percentages of sporulated cells were calculated. Results shown in Figure 12 showed no significant difference in the fraction of sporulation in *YEpCYC1-IME4-GFP* and *pJS14*; therefore, the *YEpCYC1-IME4-GFP* construct complements the mutation. The

p-value at 24 hours was 0.91 and 0.06 at 48 hours. No sporulation was noted in the *pYEpCYC1* as expected. We conclude that the *YEpCYC1-IME4-GFP* is functional.

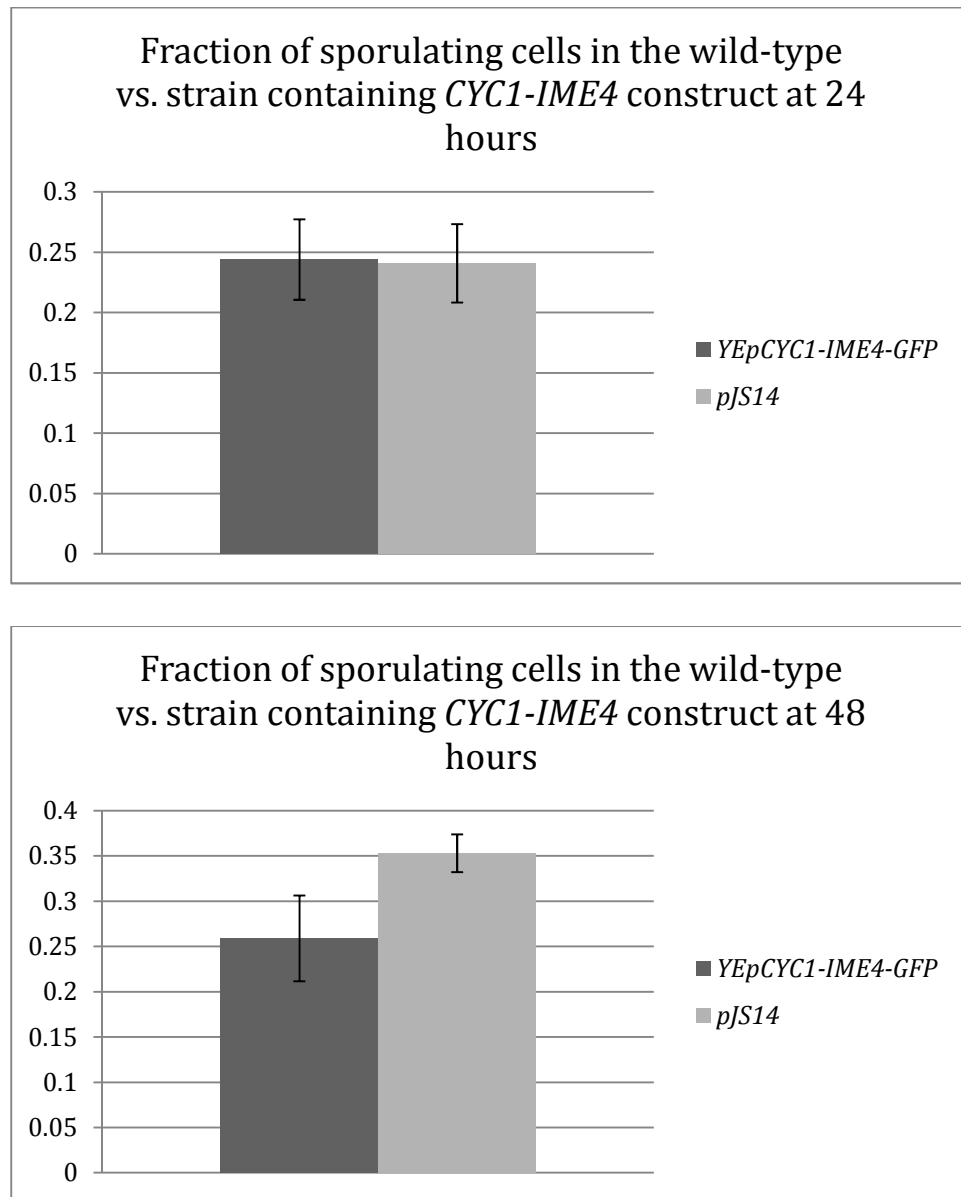


Figure 12: Quantitative analysis of the sporulation of the diploid YYF101 with plasmids *YEpCYC1-IME4-GFP*, *pJS14*, and *pYEpCYC1*. The percentage of sporulated cells was counted after incubation in SPM media for 24 and 48 hours. Error bars show standard deviation

Figure 12 (continued)

Time	Sporulation in <i>YEpCYC1-IME4-GFP</i>	Sporulation in <i>pJS14</i>	Sporulation in <i>pYEpCYC1</i>
24 hours	58/239	64/278	0/318
48 hours	70/275	88/250	0/253

Assessing Effects of Overexpression on the Mating Response

The *YEpCYC1-IME4-GFP* construct allows us to examine whether overexpression of *IME4* has any effect on the pheromone response. Strains containing *YEpCYC1-IME4-GFP* and *pYEpCYC1* were subjected to pheromone to determine any differences in the mating response in strains containing the new plasmids. Samples were taken every 30 minutes for 2 hours. An image was taken using the DIC, and the percentage of shmooing cells was determined. As expected, the percentage of shmoos increased after the addition of pheromone in both strains. No significant difference in the percentage of shmoos was noted at any time point. Therefore, both strains are capable of responding to pheromone and polarized growth to begin the mating response. Overexpression has no overt effect on the mating response (Figure 13).

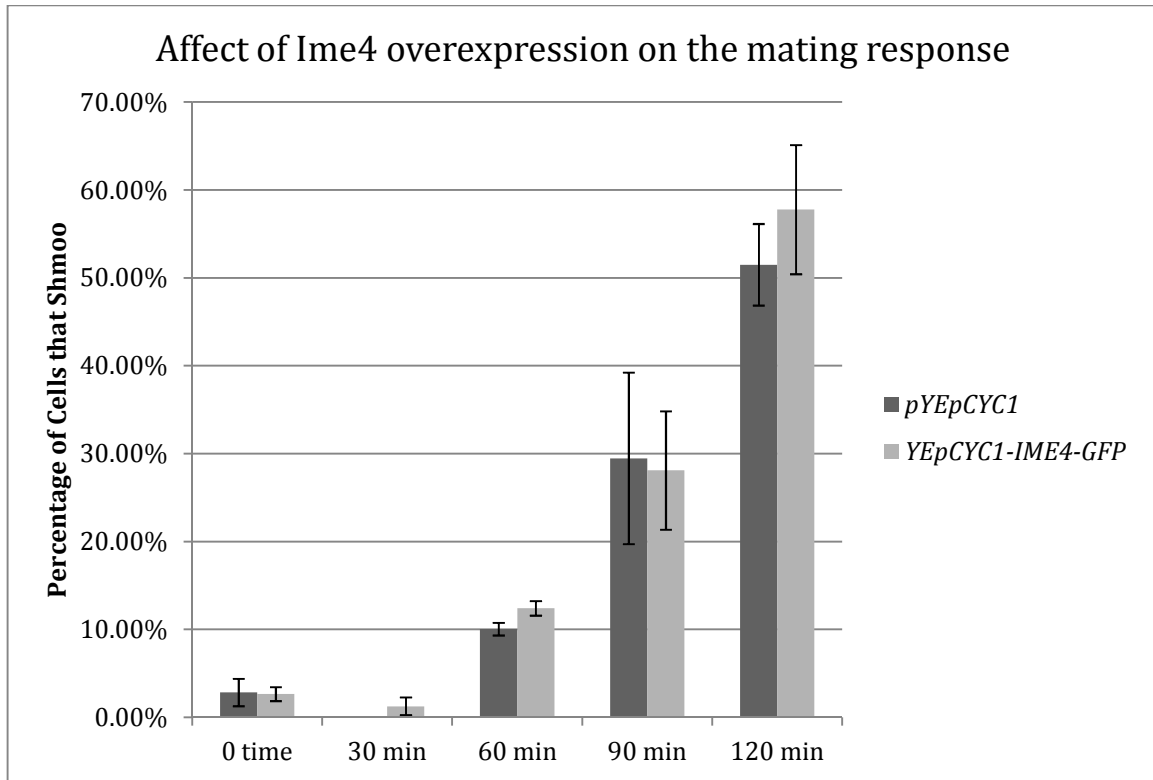


Figure 13: Quantitative analysis of the fraction of shmooed cells in strains containing *pYEpCYC1* and *YEpCYC1-IME4-GFP* at several time points following pheromone induction. Error bars showing standard deviation and p-values show no significant difference at any time point.

Pheromone Sensitivity of Yeast Strains Containing the *CYC1* Constructs

The halo assay was repeated using a strain containing new plasmid, *YEpCYC1-IME4-GFP*, and a strain containing *YEpCYC1* as a control strain to determine the sensitivities of the strains to pheromone. The strains were spread on YEPD plates, and α -factor was added to a disc of filter paper. Methanol was added as a control. The plates were grown at 30°C for 48 hours before the halo was assessed by taking an image and quantifying the diameter of the halo using Quantity One software. The average area of the halo in the yeast strain containing *YEpCYC1-IME4-GFP* cells was 237.04 mm², and the average area in yeast cells containing *YEpCYC1* was 286.68 mm². The p-value for the average areas of the halos was determined to be 0.24; therefore, no difference in pheromone sensitivity was noted between the two strains. These results, shown in Figure 14, indicate that the cell cycle arrest and recovery are unaltered by increased *IME4* expression.

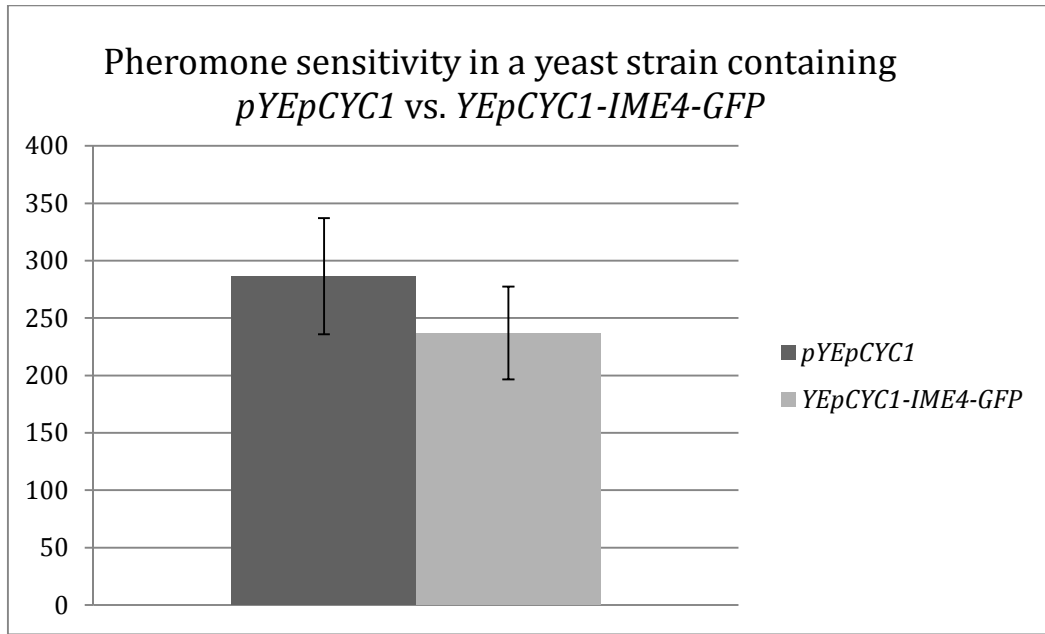


Figure 14: Quantitative analysis of halo assay of a yeast strain containing *pYEpCYC1-IME4* or *YEpCYC1-IME4-GFP* after being plated on YEPD plates with α factor in the center for 48 hours. Methanol was used as a negative control. The average area of the halo is shown with the standard deviation for error bars. The p-value is 0.24.

Nuclear Localization and Inclusion Formation by *CYC1*-Driven Ime4

To detect nuclear localization of yeast cells containing *YEpCYC1-IME4-GFP*, the strain containing the plasmid was grown at 30°C in SC casamino acids for 1 hour. Samples were then taken, mixed with ethanol, spun down and resuspended in mounting media before being stained with DAPI and viewing on the fluorescence microscope. Colocalization of Ime4 and DAPI occurred in 47.23% of 415 cells. This confirms the functionality of the *YEpCYC1-IME4-GFP* construct because the localization is the same during growth as during the pheromone response. This experiment also shows that the nuclear localization signal is constitutive; pheromone and starvation signals are not necessary to direct the protein to the nucleus. This amount of overexpression causes inclusions, which are severely aggregated proteins (Figure 15).

Nuclear localization and Inclusion Formation of *CYC1*-driven Ime4

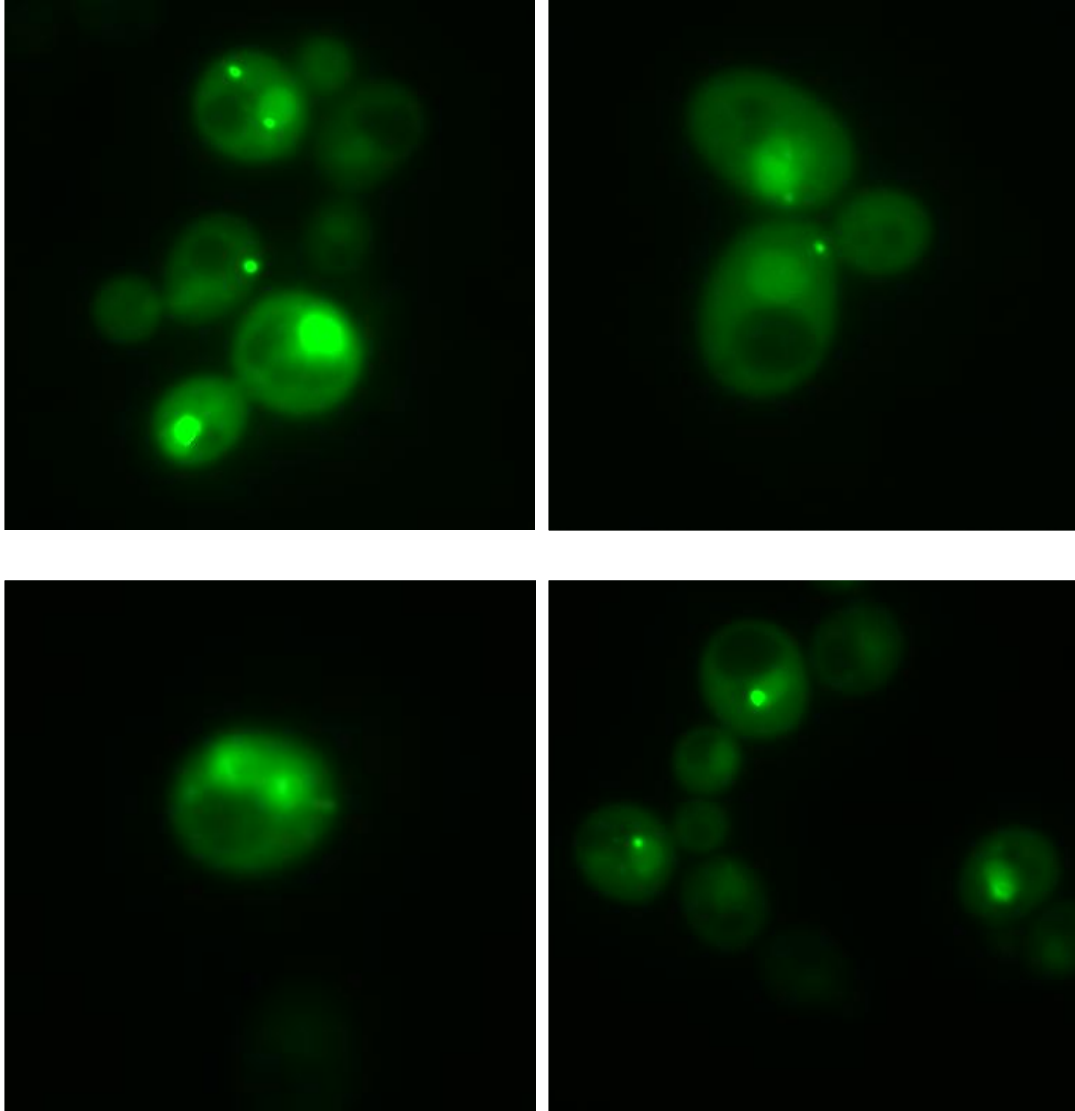


Figure 15: Ime4 aggregations in *YEpCYC-IME4-GFP* grown in SC casamino acids

Stability of Ime4 Following Cycloheximide Treatment

IME4 under the control of its own promoter is induced by pheromone but disappears after approximately 3 hours of pheromone exposure. Loss of the Ime4 signal under these conditions likely results from both reduced transcription due to Ste12 degradation, a transcription factor

used to regulate genes in mating and filamentation (Esch, 2006) and degradation of existing Ime4 protein. To assess stability of the Ime4 protein in growing cells, we examined its location and relative abundance following treatment of the cells with cycloheximide.

Cycloheximide inhibits translation elongation; therefore, strains can be treated with cycloheximide to assess protein stability and protein trafficking. A yeast strain containing *YEpCYC1-IME4-GFP* was grown for 1 hour at 30° C before the sample was split into two samples. Cycloheximide was added to one sample while the other sample was used as a control. Samples were taken every 15 minutes and viewed on the fluorescence microscope. The percentages of diffuse, cytosolic, and punctate cells were calculated (Figure 16). The only difference noted was in the percentage of punctate cells at 60 minutes and later, with the sample without cycloheximide treatment having a higher percentage of punctate cells. A nuclear and cytosolic signal was seen in all samples. These results indicate that the diffuse cytosolic signal is not solely the result of new synthesis and that the Ime4 protein is stable after synthesis. The persistence of the cytosolic signal after inhibition of protein synthesis is characteristic of actively shuttling proteins that move between the cytoplasm and the nucleus, independently of newly synthesized protein. Further experiments will be needed to test this idea rigorously.

Stability of Ime4 following cycloheximide treatment

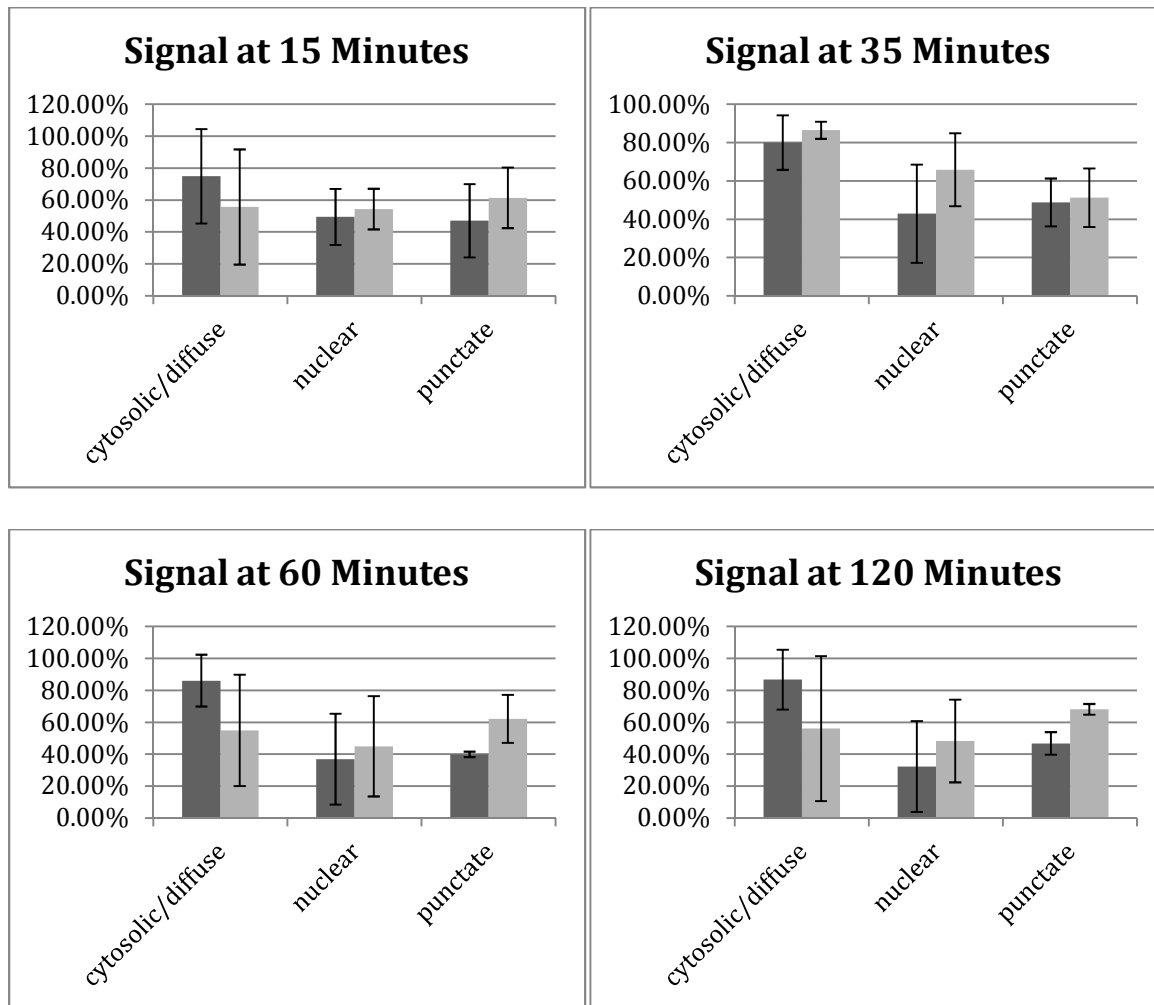


Figure 16: Quantitative analysis of the GFP-localization of a yeast strain containing *YEpCYC1-IME4-GFP* construct at various time points following cycloheximide treatment. (Dark gray: cycloheximide; light gray: no cycloheximide)

The *YEpCYC1-IME4-GFP* strain was grown in SC casamino acids for 2 hours before adding cycloheximide. Cell extracts were obtained using the “quick-kill” method of protein extraction, taking samples at various time points for 2 hours. Extracts were loaded onto and run on an SDS gel. The gel was transferred onto a PVDF membrane using the BioRad transfer apparatus and following the protocol. The primary antibody was against GFP, and the secondary

antibody, HRP-conjugated goat anti-mouse Ig. After the addition of ECL, images of the blot were taken using ChemiDoc (Figure 17). Results showed the protein was still present after 120 minutes of cycloheximide treatment, but the protein aggregated; it stayed in the wells and never entered the gel (Figure 17). Therefore, it is thought that the protein may be SDS-resistant. Phosphoglycerate kinase (PGK) was used as a loading control.

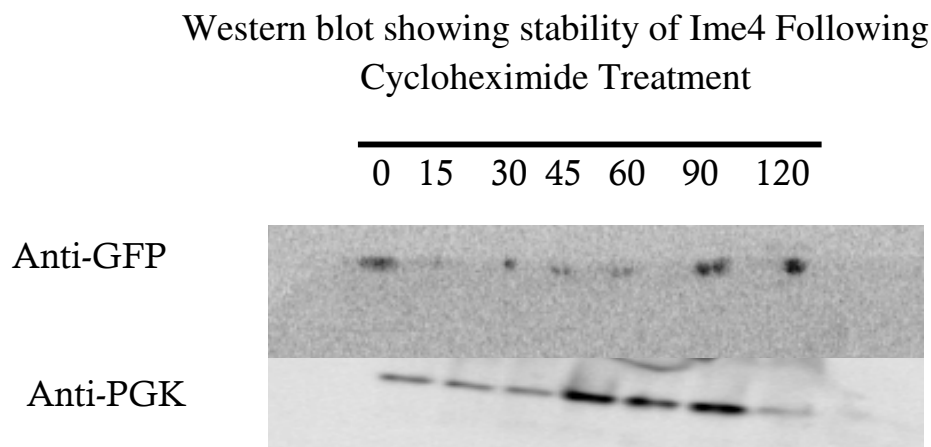


Figure 17: Western blot of *YEpCYC1-IME4-GFP* extracts at various time points following cycloheximide treatment using a primary antibody against GFP and HRP-conjugated goat anti-mouse IgG for the secondary antibody. PGK was used as a loading control.

SDS-Resistance of Ime4

To address the possibility of SDS-resistance, a different method of protein extraction and transfer known as semi-denaturing gel electrophoresis (SDD-AGE) was utilized as described by Alberti, 2009 in which no reducing agents are used in preparing the protein extracts. Once extracts were made following growth in SC liquid media, a portion was run on a 1.8% agarose gel containing 1X TAE and SDS. The gel was transferred to a membrane using the capillary transfer method, similar to the transfer utilized in Southern blots. The remaining blot procedure was done as previously. After the addition of ECL, the membrane was viewed using ChemiDoc.

Results showed a strong GFP signal at the bottom of the gel. The remainder of the protein was thought aggregate at the top, not having entered the gel.

A portion of the protein extract was boiled at different lengths and run on an SDS gel to determine if the protein could enter the gel. Results were similar to the SDS-PAGE results. The GFP portion migrated down the gel as a result of proteolysis; whereas, the Ime4 portion is thought to have never entered the gel. It was concluded that the protein is still aggregating despite the modest overexpression using the *CYC1* construct, *YEpCYC1-IME4-GFP* (Data not shown).

Mutational Analysis of Ime4 Protein

We wanted to create a form of the protein that can be utilized in biochemical approaches in a more cooperative manner. Deleting portions of the protein and analyzing its localization and ability to sporulate achieved this. In doing so, regions of the protein that are important for Ime4 function can be identified. *YEpCYC1-IME4-GFP* was used as a starting point to delete specific regions of the *IME4* gene using primers designed to achieve this. Major regions of the original *YEpCYC1-IME4-GFP* protein are depicted in Figure 18. The mutant alleles were transformed into the haploid BY4741 wild-type strain and the diploid *a/α ime4:TRP1* knockout strain, YYF101. The plasmids were constructed as a joint effort among lab members (PMD, JG, MJC). Mutations are depicted in Figure 18. We initially chose N and C terminal regions of the protein outside of the conserved MTase core that are predicted to be particularly aggregation prone by consensus methods (<http://biophysics.biol.uoa.gr>), for amyloid prediction (N-terminal), and for prion formation (QN-rich C-terminus). We also examined a region that we have defined as sufficient for binding the Ime4 pairing partner, Mum2 (S. Dahal, P. Perera, 2hybrid). None of the regions chosen for deletion included conserved MTase or MT-A70 signature regions.

Transformants were then assessed for the ability of the altered proteins to enter the nucleus, to complement the sporulation deficient phenotype of the YYF101 strain, and to form visible cytosolic aggregates. A summary of the results of this analysis is shown in Table 5.

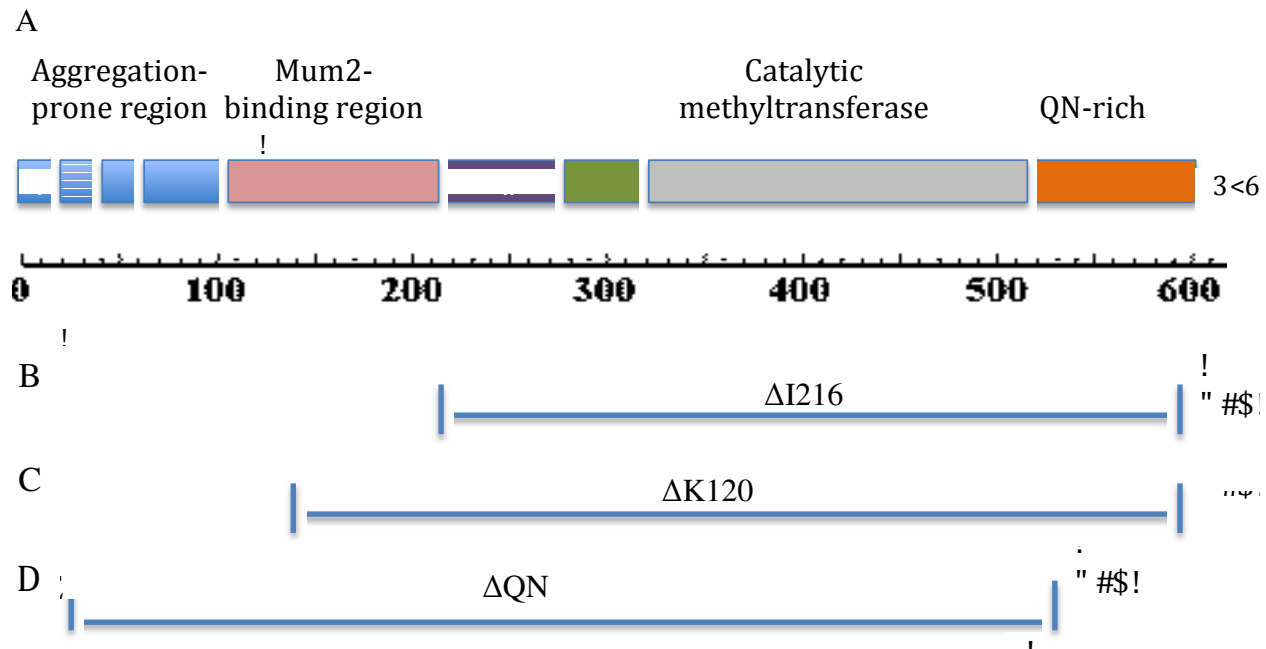


Figure 18: A. Schematic showing amino acid regions of the *YEpCYC1-IME4-GFP* construct. Blue: aggregation prone regions (1-14, 29-35, 88-108); pink: Mum2-binding domain (135-225); purple: highly conserved MT-A70 signature region (235-280); green: non-conserved loop (280-304); gray: catalytic methyltransferase domain (312-522); orange: QN-rich C-terminal domain (522-599). B. $\Delta I216$. C. $\Delta K120$. D. ΔQN

Mutation (amino acids deleted)	Nuclear Localization	Notable Features	Able to Sporulate	Formation of Aggregates
$\Delta I216$ (2-215)	Yes	Predicted aggregation-prone containing Mum2-binding	No	Yes
$\Delta K120$ (2-119)	Yes	Predicted aggregation-prone region	Yes	Yes
ΔQN (546-599)	Yes	38.2% QN, nonconserved	Yes	Yes
Full-length (1-600)	Yes		Yes	Yes

Table 5: Results of Mutational Analysis

The mutant lacking the QN-rich region (*ΔQN*) at the C-terminus, a region thought to be involved in prion formation, was transformed in BY4741 and still localized in the nucleus and formed aggregations in the cytoplasm in similar amounts as the wild-type; therefore, this region does not contain a nuclear localization signal and is not essential for the aggregation behavior of the protein. When transformed in YYF101 and grown in SPM liquid media, the cells are still able to sporulate as seen when viewed on the fluorescence microscope using the DIC filter; therefore, the region is not essential for the function of Ime4 in sporulation.

The mutant *ΔI216* lacks amino acids 2-215, which contains the aggregation-prone regions at the N-terminus as well as the Mum2-binding domain. When transformed into BY4741, the protein still localized in the nucleus similarly to the wild-type; therefore, no nuclear localization signal is present in this region. It was also still able to form aggregations in the cytoplasm as in the wild-type; therefore, this portion of the protein is not responsible for the formation of aggregates when overexpressed. When transformed into YYF101 and grown in SPM liquid media, the cells were unable to sporulate, even after 48 hours. Therefore, this region is necessary for the function of Ime4, which is likely due to the interaction with Mum2, a component of the MTase complex. Yeast 2hybrid analysis has shown that the region 135-225 is sufficient for binding Mum2.

The mutant *ΔI216* was transformed into BY4741 and used for protein extraction using the method used for SDD-AGE with a slight modification. The extract was spun down for a short time to remove debris then spun down for 10 minutes to create a pellet. The supernatant was removed, and the pellet was resuspended in lysis buffer. 1X SDS buffer was added to a portion of the extract and run on an SDS gel. The other portion was prepared as in SDD-AGE and run on a 1.8% agarose gel in 1X TE. After blotting both membranes and adding ECL, the membranes

were viewed using ChemiDoc. Results showed a band in the *ΔI216* pellet portion (Data not shown). It is concluded that the *YEpCYC1-IME4-GFP* construct forms SDS-resistant aggregates.

The mutant *ΔK120* lacked amino acids 2-119, which includes the aggregation-prone region at the N-terminus. When transformed with BY4741, grown in SC casamino acids and viewed on the fluorescence microscope, the protein still localized to the nucleus and is still able to form cytosolic aggregations as in the wild-type; therefore, this region is not necessary for nuclear localization nor aggregation. When the mutant was transformed with the diploid strain YYF101 and grown in SPM liquid media, the cells were able to sporulate similarly to the wild-type, as seen when viewed on the fluorescence microscope using the DIC filter. Therefore, the function of Ime4 is unaltered in this mutant. This is likely due to the fact that the Mum2-binding region is still present; therefore, the MIS complex is still able to form in order to function.

DISCUSSION

Roles of Ime4 in Pheromone Response

Ime4 is an inducer of meiosis that is expressed in diploid yeast cells and has a primary role in sporulation. Recent data has shown that it may also have a role in mating in haploid cells. Confirming this result, a Western blot using extracts of haploid cells that have been exposed to alpha factor showed an increase in Ime4 protein expression (Figure 1). Therefore, Ime4 is induced in response to pheromone, although its role is unknown. This pheromone-induced expression suggested that Ime4 might play a role in mating. Specifically, two independent studies demonstrated that the *IME4* mRNA is induced by pheromone. However, the sensitivities of wild-type and an yeast strain containing an *ime4* mutant to pheromone show no significant difference. This was noted when analyzing the ability of the two strains to shmoo after pheromone was introduced and also in the halo assay; the halos were similar in both strains as a result of the ability to halt the life cycle (Figure 2). No difference in halo size was seen when comparing yeast strains containing *YE_pCYC1-IME4-GFP* or *pYE_pCYC1* exposed to pheromone (Figure 14). Because no difference in the size of the halos was noted between the mutant and wild-type strains, Ime4 is not thought to have a role in cell cycle arrest or recovery from pheromone.

Even though no obvious differences were seen in the halo or shmoos, this does not rule out other factors of mating that were not tested. For instance, overall mating efficiency and the ability to form diploids was not tested. Ime4 could have roles in later events in the mating process, such as cell fusion or nuclear fusion. Indeed, *IME4* is a member of the pheromone-induced *KAR4*-dependent cluster of genes that require higher levels of pheromone and that occur somewhat later in the pathway. Another gene in this cluster includes *KAR3*, which is needed for

nuclear migration but not for shmoo formation or resumption of the cell cycle. For others, like *IME4*, the function is not known. Very few genes are induced by both sporulation and pheromone, and these induce both *IME4* and its distant paralog, *KAR4*.

Ime4 could also have a redundant role with another protein in which either protein is sufficient. For example, Mpt5 regulates translation of some RNAs to cause attenuation by binding to specific sites within the 3' UTR. Ime4 could also cause attenuation, although either protein is adequate to cause a response. If a protein is thought to have a redundant function, a double knockout can be made to determine if this has any effect on mating.

Another aspect to test for is the idea of bilateral mating defects in which detrimental effects are only seen when genetic defects are present in both mating partners. Some mutants exhibit severe mating defects only if they are mated with a strain with the same mutant or a mutant with a similar function. An example is strains that are defective in *FUS1* and *FUS2* genes. Defects are not noted when mated with a wild-type strain, but they are seen when mated with a strain that also has defective *FUS1* and *FUS2* genes (Guthrie, 2004). Therefore, the effects of an *ime4* mutant may not be seen unless two mutants are allowed to mate.

Although mRNAs have been shown to be methylated by Ime4 during sporulation conditions, this has not been shown following pheromone induction during mating. One way to show this is using thin layer chromatography as described by Bodi (2010). mRNA that has been extracted following treatment with pheromone can be digested with T1 ribonuclease, which digests the mRNA after guanosines, which is where m6A is noted most commonly. The products are then end labeled, and subjected to thin layer chromatography (TLC). This will give a quantitative value of m6A compared to A. Another method to identify m6A is by immunoprecipitation as described by Bodi (2010). A mouse monoclonal anti-m6A antibody that

recognizes m6A in single stranded nucleic acids can be used. RT-qPCR can then be used to quantify the amount of methylated mRNA. We conclude that Ime4 is induced during mating, although its role is still unknown.

Nuclear Localization of Ime4

When yeast strains containing Ime4-GFP were allowed to grow and induced, the GFP signal was noted in the nucleus at some points as seen when viewed on the fluorescence microscope following DAPI staining (Figures 5 and 15). Nuclear localization was noted in both strains containing the plasmid with the native promoter and induced with pheromone (Figure 5) as well as strains containing the plasmid construct containing the *CYC1* promoter, *YEpCYC1-IME4-GFP* (Figure 15). This is in agreement with studies done on mammalian, plant, and insect (*Drosophila*) in which Ime4 orthologs are nuclear, specifically in splicing-associated “speckle” regions (Bokar, 1997; Hongay, 2011; Zhong, 2008).

Mutational Analysis to Identify a Nuclear Localization Signal

Because some of the Ime4 protein is nuclear and is expected to function there, we inspected the amino acid sequence for potential nuclear localization signals (Appendix). Mutational analysis was undergone in which regions of the protein likely to contain a nuclear localization signal were removed. The plasmids *ΔI216*, *ΔK120*, and *ΔQN* were transformed into the haploid BY4741, grown in liquid media and viewed on the fluorescence microscope. The protein was still localized to the nucleus; therefore, The N-terminal region of amino acids 2-215 and the C-terminal region of amino acids 546-599 do not contain a nuclear localization signal. As a result, we conclude that sequences essential for nuclear localization are located within the ~300 amino acid region that comprises the conserved core of this protein (Figure 18A).

Protein Trafficking and Nuclear Localization Signals

In eukaryotes, a nuclear envelope defines the nucleus and separates it from the cytoplasm. This allows regulation for expression of proteins and RNA. Proteins are made in the cytoplasm and could have a role in the nucleus, and RNA is made in the nucleus and needs to be transported to the cytoplasm to be translated. The passage of molecules between the nucleus and the cytoplasm is through the nuclear pore complex, which is where the inner and outer membranes of the nuclear envelope are joined. Larger macromolecules, such as proteins and RNA, pass through actively and selectively. Proteins that are targeted to the nucleus have a nuclear localization signal, a specific amino acid sequence in many cases composed of basic amino acids, which is recognized by a nuclear transport receptor known as an importin. From here, proteins can stay in the nucleus or be shuttled back and forth between the nucleus and the cytoplasm. Proteins going back into the cytoplasm have a nuclear export signal that is recognized by a receptor known as an exportin. Importins and exportins are members of the family of nuclear transport receptors known as karyopherins. The different importins and exportins bind to a specific cellular component to aid in trafficking. For example, the importin Kap α /Kap β 1 dimer binds proteins with the “classic” basic amino acid nuclear localization signal (Cooper and Hausman, 2010), and Los1p binds tRNAs to allow their export from the nucleus (Sarkar, 1998). Regulation of nuclear import can be controlled by other proteins that bind and mask the nuclear localization signal or through phosphorylation (Cooper and Hausman, 2010).

The best-understood (a.k.a. “classic”) nuclear localization signals contain positively charged residues. Monopartite nuclear localization signals contain basic residues preceded by a helix-breaking residue; the consensus sequence, first identified in the SV40 Large T-antigen, is PKKKRKV. A bipartite nuclear localization signal contains two sets of basic residues separated

by a spacer of about 9-12 amino acids and has the consensus sequence KR[PAATKKAGQA]KKKK. Importin α recognizes the classical nuclear localization sequence, whereas importin β directs the translocation from the cytoplasm to the nucleus via the nuclear pore complex. Once inside the nucleus, the cargo is released, and importin α is recycled back into the cytoplasm to be reused (Lange, 2010). The human protein, MT-A70 contains two potential nuclear localization signals within the N-terminal portion of the protein, a region that is not conserved across lineages, but neither sequence has been directly shown to function as a nuclear localization signal (Bokar, 1997). Ime4 and its fungal orthologs lack any obvious matches to the classic localization signals, suggesting that a different mechanism is used to direct these proteins to the nucleus.

In addition to the classical nuclear localization signal, non-classical nuclear localization signals also exist, although few have been characterized in detail. One such class is the PY nuclear localization class, which is recognized by karyopherin $\beta 2$ (Kap104 in yeast) (Lee DCY and Aitchison, 1999). Signals that bind to Kap β have three notable characteristics. The signals are structurally disordered when not bound to Kap β . The nuclear localization signal has an overall positive charge because the substrate-binding domain of Kap β is acidic. The consensus motif consists of a hydrophobic or basic motif followed by the C-terminal motif R/K/HX₍₂₋₅₎PY, where X is any amino acid (Lee, 2006). In yeast, the mRNA-binding protein Hrp1 has been found to contain a PY-NLS-like sequence that is necessary for its nuclear import (Lange, 2008). A possible PY nuclear localization signal exists in the Ime4 protein, although its localization is in the core catalytic domain spanning the catalytic motif IV MTase signature sequence. For this reason, we believed this to be the functional nuclear localization signal. On the other hand, this study demonstrated that neither the first 215 nor the last 54 amino acids are necessary for nuclear

localization of this protein. Further research is necessary to determine if the non-classical PY nuclear localization signal is present, which may involve mutagenesis of the PY motif, a very highly conserved motif among most *IME4* family members. Mutagenesis of several basic patches within the conserved domain can also be used to determine possible nuclear localization signals.

Another type of nuclear localization signal involves basic amino acids within the zinc finger region, such as the signal identified in TR2 in mice (Yu, 1998). Ime4 includes a cysteine-rich region that is likely to be a metal-binding domain, although the pattern does not conform to any known zinc-binding structures. It is possible that basic residues within this region define the nuclear localization signal.

Ime4 as a Stable Shuttling Protein

To be functional, cellular components that transport need to coordinate between the nucleus and the cytoplasm. For example, RNA-binding proteins that facilitate the transport of mRNPs often shuttle between the nucleus and the cytoplasm; they bind the mRNP in the nucleus for its transport, undock in the cytoplasm, and transport back across the nuclear membrane into the nucleus. Other proteins, by contrast, are nuclear-resident, remaining in the nucleus for their function. Microscopy studies in pheromone treated cells and in the strain containing *YEpCYC1-IME4-GFP* show that Ime4 is present in both the nucleus and the cytoplasm. This lead to the possibility that Ime4 could shuttle between the nucleus and the cytoplasm with roles in each.

One way to detect if proteins shuttle between the nucleus and the cytoplasm is by treatment with cycloheximide. Cycloheximide halts protein synthesis; therefore, no new proteins are made. Proteins will then either move to the nucleus or cytoplasm for the duration of their function or shuttle between the nucleus and the cytoplasm if they are shuttling proteins. The

balance of the localization of shuttling proteins in the cytoplasm and nucleus and the rate of transport may depend on the cellular environment. For example, the shuttling protein Rpb4p exports from the nucleus faster during stressful conditions (Selitrennik, 2006).

In these experiments, a yeast strain containing *YEpCYC1-IME4-GFP* was treated with cycloheximide, and GFP was still noted in both the nucleus and the cytoplasm (Figure 16). The only significant difference noted was more cells with Ime4 aggregates in cells not treated with cycloheximide in samples taken after 60 minutes. Overall, Ime4 is noted to be a stable protein with a nuclear and cytosolic signal that is not exclusively a result of new protein synthesis. Although this suggests shuttling, it is not proven; GFP could give signal by itself after proteolysis. Mutants can be constructed to possibly resolve this issue.

Several genetically-based nuclear shuttling assays have been devised to answer this question. Because nuclear localization signals contain residues that are positive, site-directed mutagenesis on the positive residues can be used to disrupt the nuclear localization signal if present (Lange, 2006). Another method to detect if a protein shuttles is by using a strain that contains a temperature sensitive mutant for the export receptor Crm1/Xpo1 (Vergara, 2010). Shuttling proteins will accumulate in the nucleus and will be unable to perform its role in the cytoplasm.

Protein Inclusion Formation and Possible Cytosolic Roles of Ime4

In yeast cells containing *IME4-GFP* treated with pheromone or containing *YEpCYC1-IME4-GFP* cells, the protein aggregates when expressed for a long period as seen using fluorescence microscopy (Figure 15). The types of inclusions that can form are numerous, including P-bodies, stress granules, aggresomes (JUNQ compartment), IPODs (insoluble protein deposit), and prion-like amyloid aggregates, among other types of ill-defined foci.

P-bodies, Stress Granules, and Other Cytosolic Foci

To maintain protein homeostasis, proteins are refolded, degraded, or sequestered into protein aggregations to prevent improper interactions. Chaperones are at the center of regulation. They are able to assist in refolding, target proteins for degradation, or target proteins for inclusion formation. Misfolded proteins can form inclusions to separate them spatially and functionally from functional proteins. Inclusions can have aggregated proteins that are soluble or insoluble. The aggresome is an insoluble structure that colocalizes with the microtubule organizing center; therefore, it is a perinuclear structure. The JUNQ (juxtannuclear quality control), which is likely the same as the aggresome, is a soluble aggregation of misfolded proteins located on the cytosolic surface of the endoplasmic reticulum. Once aggregated, the protein can be refolded or targeted for degradation through the ubiquitin-proteasome pathway. The IPOD is an insoluble protein aggregate (Chen, 2011).

P-bodies and Stress Granules

Expression of mRNA is in part controlled by the regulation of mRNA turnover. This involves deadenylation of the poly-A tail, removal of the 5' cap, and degradation of the transcript by 5' to 3' exonucleases. P-bodies are processing bodies, recognized in the cells of yeast and mammals as discrete cytoplasmic foci that contain enzymes for mRNA degradation. For instance, Dcp1 and Dcp2 are decapping enzymes, Xrn1p is a 5' to 3' exonuclease, and Dhh1p is in the helicase family of proteins and is involved in the activation of decapping (Teixeira, 2005). P-bodies have also been noted to have roles in nonsense-mediated decay, mRNA storage, and translation repression (Buchan, 2008). They increase in size and decrease in number during times of stress; for instance, later stages in growth, glucose deprivation, osmotic stress, and UV light exposure. Because P-bodies are associated with enzymes involved in translation repression, P-

bodies and translation have a reciprocal relationship; P-bodies increase at times when translation is limiting. During translation, mRNA is trapped in the polysome; therefore, they are unable to aggregate in order for P-bodies to form (Teixeira, 2005). Rpb4p is a component of RNA polymerase II and associates with nascent RNA during transcription. The shuttling protein Rpb4p binds mRNA in the nucleus and transports it into the cytoplasm for another role. In some cases, mRNA bound to Rpb4p associates with P-body components for degradation of the mRNA. Once the mRNA is degraded, Rpb4p returns to the nucleus for another round (Selitrennik, 2006).

Many P-body components have regions that are rich in glutamine (Q) and asparagine (N), such as the Lsm4 protein, which co-localizes with other P-body components during stress and has a highly conserved Q/N rich region in the C-terminal domain that is necessary for RNA degradation. The region is thought to assist in aggregation with proteins with similar regions, leading to localization in P-bodies. The conserved region present in many proteins is thought to also have a role in prion formation in addition to P-body assembly (Reijns, 2008).

Stress granules are similar to P-bodies in that they are cytoplasmic particles, containing nontranslating mRNA, that form during stress. Stress granules and P-bodies are also able to dock and undock from each other under certain conditions. Stress granules differ in that they are only present during stress and are dependent on P-bodies to form, whereas P-bodies form independently and can be present at all times, but increase during stress. Stress granules contain translation initiation factors, such as the 40S ribosomal subunit and the poly-A binding protein; therefore, it may be a site of translation re-initiation.

The process of P-body and stress granule interaction begins with mRNA that is associated with the polysome and being translated into protein. When introduced to stress,

premature termination of translation results. This causes the partially translated mRNA to leave polysome and aggregate with P-body components. At this point, the nontranslating mRNA has two fates. It can be decapped and degraded by the P-body components. Another possibility is the transition of the P-body into a stress granule. The P-body components leave and the translation initiation factors are loaded to form a stress granule. Once conditions are favorable, the partially translated mRNA can re-enter translation (Buchan, 2008).

The possibility of Ime4 aggregating into P-bodies was assessed through various experiments. Co-localization of Ime4-GFP with Dcp2-RFP was determined using fluorescence microscopy. Results showed some co-localization, although the majority of Ime4 aggregates did not co-localize with P-bodies (Figure 7). By using plasmids containing a mutant in the P-body component *edc3*, it was determined that Ime4 aggregates do not rely on the presence of P-bodies to form (Figure 9). The number of Ime4 aggregates was similar in the wild-type BY4741 and strain that is unable to form P-bodies well. Also, P-bodies form in similar quantities when Ime4 is overexpressed as seen when comparing the number of cells able to form P-bodies in a strain containing *pJS14* and a strain containing just the vector, *YEp352* (Figure 10). Therefore, P-bodies do not rely on Ime4 aggregates to form. It is concluded that Ime4 aggregates into another type of inclusion other than P-bodies.

A similar method can be used to detect co-localization of stress granules with Ime4. A stress granule component, such as Pabp, can be tagged with RFP as in Buchan (2008), transformed with a strain containing *IME4-GFP*, and grown in liquid media under conditions that induce both *IME4* and stress granule formation. Samples can be viewed on the fluorescence microscope for the presence and co-localization of both GFP and RFP. Also, a mutant of a stress

granule component can be constructed and transformed with a strain containing *IME4-GFP*, and the ability to form Ime4 aggregations can be assessed using fluorescence microscopy.

Prions

The work in this thesis demonstrates that overexpressed Ime4 forms insoluble aggregates that are prion-like in that they are SDS-resistant and insoluble. A prion can be defined as a protein that is able to convert between structurally and functionally distinct states, some of which are transmissible. The classic prion is Sup35 and all others are compared to this. Prion-forming domains (PrDs) are rich in glutamine and asparagine and can be transferred to other proteins to create new prions. This does not define a prion because some proteins have regions rich in glutamine and asparagine and are unable to form prions (Alberti, 2009). Sequencing of *IME4* reveals a region rich in glutamine and asparagine in the C-terminal region. Mutational analysis in which this region was removed, mutant ΔQN , showed Ime4 aggregates to still form when transformed into BY4741 and grown in SC casamino acids. Therefore, this region is not responsible for forming prions if they exist in Ime4. Once the PrD is established, it is able to self-propagate. Some prions have an insoluble aggregated state composed of an amyloid-like structure, containing highly ordered beta sheets.

The switch to the prion state is noted more during stressful situations. In yeast, prions are linked to gene expression; therefore, prion proteins are thought to act as an epigenetic switch to regulate pathways involved in the stress response pathway (Alberti, 2009). Many more proteins are evident that can act as prions than have been anticipated, including RNA-modifying proteins (e.g. MOD5), transcription factors (e.g. CYC81 and SSNG) and RNA-binding proteins (e.g. Lsm4 and Puf2) (Reijns, 2008).

Because of the aggregations, SDS-PAGE is not useful to view these proteins; when a western blot was done, the protein never left the wells (Figure 17). Therefore, SDD-AGE can be used to resolve SDS-resistant aggregates. In this case, protein extracts are made using protease inhibitors and no reducing agent, and an agarose gel is used instead of an SDS gel. Those rich in asparagine are more likely to form amyloid aggregates, whereas those rich in glutamine are non-amyloid aggregates that cannot be detected by SDD-AGE (Alberti, 2009). When the protein extracts were subjected to SDD-AGE, the pellet of the *ΔI216* protein extraction containing a mutation in which the aggregation-prone region, including the Mum2 binding domain, was removed, was visible on the membrane following a Western blot, indication that by this functional definition, Ime4 is prion-like (Data not shown).

JUNQ and IPOD

Other possibilities for protein aggregates are sequestration into an IPOD (insoluble protein deposit) or JUNQ (juxtannuclear quality control), most likely the same as an aggresome. The JUNQ and IPOD are thought to have roles in protein quality control during times of stress when there is an abundance of misfolded proteins. Misfolded proteins sequester in these components before being degraded or refolded. The JUNQ is located on the cytosolic surface of the endoplasmic reticulum and contains proteasomes and chaperones to increase clearance. Soluble misfolded proteins aggregate in the JUNQ before being degraded or refolded depending on whether the protein has been ubiquitinated. Insoluble proteins, such as huntingtin and prions, aggregate in the IPOD and do not associate with proteasomes. The IPOD is thought to have a role in sequestering insoluble proteins during stress so they do not occupy the proteasome when other proteins need to be degraded (Chen, 2011).

The various mutants created in which aggregation-prone regions were deleted still aggregated as seen when the plasmids were transformed with BY4741, grown in SC casamino acids, and viewed using fluorescence microscopy. This includes *ΔQN* in which the glutamine and asparagine-rich region was removed, *ΔI216* in which the N-terminal aggregation-prone and the Mum2-binding regions were removed, and *ΔK120* in which the N-terminal aggregation-prone region was removed. Therefore, these regions are not important in the aggregation properties of Ime4.

To further test aggregation of Ime4 into the JUNQ compartment or IPOD, an approach using co-localization of tagged proteins can be used. The Hsp104 protein localizes to both the IPOD and the JUNQ. To test for co-localization with the JUNQ in particular, the endoplasmic reticulum marker Sec63 can be used because the JUNQ has shown to be localized in an indentation in the nucleus and next to the endoplasmic reticulum. A tagged autophagy marker Atg8, which is found to be evident in the IPOD, can be used to assess co-localization of Ime4 with the IPOD (Kaganovich, 2008).

Possible Role of Ime4 in Splicing

Previous studies have shown that MT-A70 could have a role in alternative splicing or increase the efficiency of splicing introns. Yeast cells only have about 300 genes with introns, the majority of which are ribosomal protein genes and genes induced during meiosis. *MER1* is a gene that was first recognized to have a role in spore viability, meiotic recombination, and formation of the synapse during meiosis, but it is actually an RNA-binding splicing factor. Deletion of *MER1* reduces the splicing efficiency of four introns in the yeast genome that are involved in meiosis. *MER1* is controlled by the transcription factor Ume6p; splicing by Mer1 indirectly activates the transcription factor Ndt80p and thereby leads to progression into meiosis.

This method is thought to act as another level of temporal control of expression of genes during meiosis; control is both transcriptional and through splicing (Munding, 2010). One could test whether methylation by Ime4 is important for splicing to occur using whole genome splicing-sensitive microarray analyses as explained by Munding (2010). The intron accumulation indexes can be compared between the wild-type and mutant *ime4* at different time points during meiosis. Although a role of m6A in splicing in yeast has been suggested, the transcripts that Ime4 is known to methylate, *IME1*, *IME2*, and *IME4*, are not spliced; therefore, it is not likely that the primary role of mRNA methylation by Ime4 is in splicing (Bodi, 2010).

Conclusion

Ime4 is induced during mating in haploids. Its role is not known, although a role in cell cycle arrest and recovery from pheromone is not likely unless its role is redundant with another protein. Ime4 localizes to the nucleus at some points; a nuclear localization signal has not been identified, but amino acids 2-215 and 546-599 do not contain a signal as seen through mutational analysis. A possible non-classical PY nuclear localization signal exists, but it is present in the core catalytic region of the protein. Cycloheximide treatment suggests Ime4 is a stable shuttling protein whose nuclear localization is not solely the result of newly synthesized protein. When highly expressed for a longer period, Ime4 forms cytosolic foci, which could be categorized as P-bodies, stress granules, prions, or sequestered in the JUNQ or IPOD. Ime4 aggregates are unlikely P-bodies. They show some characteristics of prions, such as SDS-resistance, but prion formation has not been proven. Future research must be done to determine localization of Ime4 into stress granules, prions, IPOD, and JUNQ.

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APPENDIX

Figure 1: Amino Acid Sequence of Ime4

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ORIGIN  
1   mindklvhfl iqnyddilra plsgqlkdvy slyisggydd emqklrndkd evlqfeqfwn  
61  dlqdiifatp qsiqfdqnll vadrpekivy ldvfslkily nkfhafyytl ksssssceek  
121 vsslttkpea dsekqllgr llgvlnwdvn vsnqglpreq lsnrlqnllr ekpssfqlak  
181 erakyttevi eyipicsdys hasllstsvy ivnnkivslq wskisacqen hpqlieciqs  
241 kihfipnikp qtdislgdcs yldtchklnl cryihylqyi psclqeradr etasenkrir  
301 snvsipfyt1 gnscsahcikk alpaqwircd vrkfdfrvlq kfsvviadpa wnihmnlpyg  
361 tcndiellgl plhelqdegi iflwvtgrai elgkeslnnw gynvinevsw iktnqlgrti  
421 vtgrtghwln hskehllvgl kgnpkwinkh idvdlivsmr retsrkpdel ygiaerlagt  
481 harkleifgr dhntrpgwft ignqltgnci yemdverkyq efmksktgts htgtkkidkk  
541 qpsklqqqhq qqywnnmdmg sgkyyaekq nqmngkhtpf eskqqkqqf qtlennlyfaq  
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VITA

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